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From the Department of Physiology and Medical Biophysics Biomed cal Center,
University of Uppsala Sweden

Intracellular Accumulation and Permeability Effects of Some Weak Acids in the Isolated Frog Gastric Mucosa

By

GUNNAR FLEMSTROM

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Abstract

FLEMSTROM G *Intracellular accumulation and permeability effects of some weak acids in the isolated frog gastric mucosa* Acta physiol scand 1971 82 1—16

the ionic permeability of the frog gastric mucosa. A similar effect was obtained with acetic acid It is probable that intracellular accumulation of undissociated weak acids is the mechanism underlying their permeability increasing effects on the gastric mucosa.

Absorption of weak acids from the gastric lumen has many impo and pharmacological aspects and has attracted much interest duri Teorell (1939) showed that undissociated weak acids disappe gastric lumen with a greater velocity than their dissociated anions of disappearance was considerable for both It has since been den by several authors that weak acid are more quickly absorbed from when undissociated in acid solution (for a review see Schanker 19 suggested that an undissociated weak acid could diffuse from th the muscosal cells and that intracellular accumulation would dissociated anion at the higher intracellular pH would be un the cell membranes as fast as the undissociated acid No experi provided however

Some weak acids have been shown to increase the ionic p mucosa not only during an instillation of the undissoci but also on subsequent instillation of h drochloric acid found on instillation of 170 meq/l acetic acid into th Frenning and Öbrink 1964) and n instillat on of acetic salicylic acid into the dog stomach pouch Davenp

anions of the same acids had no effects on the mucosal permeability (Davenport 1964, 1967, Flemström and Frenning 1968). Davenport (1964, 1967) suggested the permeability increasing effects of undissociated acids to be due to persistent cellular shedding and injury of the mucosa. Flemström and Frenning (1968), however showed that the increase in ion permeability after an installation of 170 meq/l acetic acid lasted for no longer than 90 min. They therefore suggested that the permeability increasing effects might be due to an accumulation in the gastric mucosal cells of the migrating undissociated weak acid transiently persistent after removal of the acid.

In view of the different rates of absorption from the gastric lumen and the different permeability effects it seemed of interest to gain further information about the mechanisms of migration of undissociated weak acids and their anions in the gastric mucosa. The isolated frog gastric mucosa was considered suitable for this study as some short- and medium-chain fatty acids are metabolized by this preparation and increase its rate of hydrogen ion secretion (Alonso *et al.* 1967). The rate of migration of these acids or their anions from the solution on the mucosal (secretory) side into the cells might thus be estimated from the rate of secretion. Acetate and propionate, two short-chain fatty acids, and L-lactate and D-lactate were used.

Lactic acid dehydrogenase, which converts lactate to pyruvate, is strictly stereospecific. In vertebrates only the L-specific enzyme has been shown to exist (Long and Kaplan 1968). Thus the use of L-lactate and D-lactate, only the former of which is a possible substrate, permitted comparison of the effects of two weak acids with almost identical physical properties on hydrogen ion secretion.

Methods and Materials

Frogs of the *Rana temporaria* species were used. They were kept in a tank containing tap water at a temperature of 6—10 °C. Each animal was given approximately 125 mg of liver once or twice a week by forced manual feeding. No food was given for two days preceding an experiment.

After decapitation and pithing of the animal, the stomach was removed and opened along the lesser curvature. The mucosa was separated from the rest of the stomach wall by blunt dissection in an oxygenated and buffered frog Ringer solution. It was then mounted as a membrane between two compartments of a perspex chamber with an exposed area of 1.8 cm². The time necessary for dissection and mounting did not, as a rule, exceed 6 min. The chamber, the connection tubes and the reservoir contained 20 ml of solution on each side. A flow of gas through small polythene tubes on each side gave a satisfactory circulation of the solutions. The gas on the mucosal side contained 100 % O_2 and that on the serosal side 95 % O_2 and 5 % CO_2. All experiments were performed at [illegible]0 ± 0.1 °C (Thermostat F. Haake Gebr. K. G. Berlin).

After the mounting procedure the solutions in the chambers were changed 3—4 times during a 20 min period before the experiments were started. Variations in the composition of the solutions during an experiment were obtained by exchange of the solutions.

The rate of active charge transport was measured by the short-circuit technique developed by Ussing and Zerahn (1951). The transmucosal electric potential difference was reduced to zero in all experiments by current applied to the mucosa from an external source through two flat Ag-AgCl electrodes with the same diameter as that of the exposed mucosal preparation. The magnitude of the current was determined from the voltage drop over a 1 kΩ precision resistor. Continual adjustment of the current was performed manually. The Ag-AgCl electrodes were separated from the circulating solutions by a thick cellophane membrane in each compartment. The solution on the electrode side of one cellophane membrane was always the same

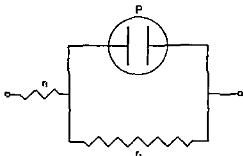


Fig 1 Electric equivalent circuit for the frog gastric mucosa

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The *resistance* of the mucosa was estimated after every determination of the electric potential difference from the equilibrium change of the open circuit potential when a fixed current (30 $\mu A/cm^2$) was passed through the mucosa in the same direction as the short-circuit current After correction for the resistance of the solutions (20—25 ohm) it was calculated by dividing the potential change by the magnitude of the fixed current

semicircle and that the mucosa can be approximately represented by the electric equivalent circuit shown in Fig 1, where r_1 and r_2 are pure ohmic resistances and P represents the "polarization capacity"

An impedance locus diagram and the symbols used are given in Fig 2 According to Cole (1940), Teorell and Wersall (1945) and Schwan and Cole (1960), R_∞ (r_1) corresponds to the resistance of electrolytes outside and inside the cell $R_0—R_\infty$ (r_2) is the resistance lying parallel to the "polarization capacity" It gives a qualitative indication of the ionic permeability

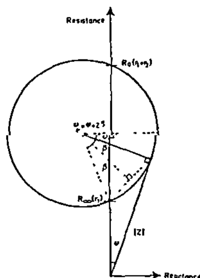


Fig 2 An ...

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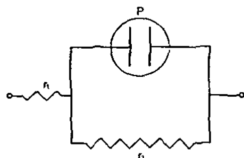


Fig 1 Electric equivalent circuit for the frog gastric mucosa

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To obtain more complete information about the electric properties of the mucosa *impedance measurements by alternating currents* were made in some experiments A modification of the method and the formulism of Teorell and Wersall (1945) were used These authors demonstrated that the impedance diagram in the fully oxygenated frog gastric mucosa is part of a semicircle and that the mucosa can be approximately represented by the electric equivalent circuit shown in Fig 1, where r_1 and r_2 are pure ohmic resistances and P represents the polarization capacity

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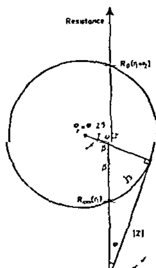


Fig 2 An impedance locus diagram R_0 is the low frequency resistance R_∞ the high frequency resistance φ the phase angle and φ_p the polarization angle |Z| is the impedance p is an angle used for the calculations

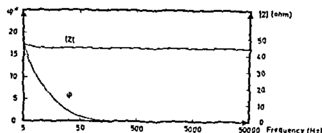


Fig 3 The impedance (|Z|) and the phase angle (φ) of the impedance electrodes and the solution between them determined with the mucosal and the serosal side solutions mixed and the mucosa absent

of the tissue membranes such that an increase indicates a decrease of the permeability The polarization angle (φ_p) gives information about the ion impermeable structures containing the *polarization capacity* (*P*) *P is to a great extent dependant on the capacitive properties of* double layer membranes and other polar structures in the tissue

The phase angle and the impedance in the frequency range 20 *Hz*—50 *kHz* were determined by a vector impedance meter (Hewlett Packard 4800 A) with a test signal of less than 2 2 mV for impedances below 1000 ohm and recorded on an X Y recorder (Hewlett Packard 7035 B) The time required for an impedance run was 2—3 min These impedance measurements were performed when the transmucosal electric potential difference was reduced to zero by the (direct) current applied for measurement of charge transport The resistance of the external d c circuit was > 200 kohm to avoid influence on the a c determinations

The electrodes for measurements with alternating currents were made from platinum net (Unimesh 48) and located 5 mm from the mucosal surface on each side The diameter of these electrodes was the same as that of the exposed mucosa They were platinized according to Jones and Bollinger (1935) to minimal polarization Fig 3 shows the impedance |Z| and the phase angle (φ) of the electrodes and the solution between them determined with the mucosa absent In the frequency range employed the influence of electrode polarization on the determinations of mucosal impedance was negligible

The parallel resistance (r_2) the polarization angle (φ_p) and the radius of the locus diagram semicircle (Rad) could be calculated rapidly from the recordings as the impedance vector [illegible]s the tangent of the locus diagram semicircle when φ is maximal (*cf* Fig 2) Using the [illegible]lues of this vector and the high frequency resistance (r_1) the following equations may be [illegible]ained

$$r_2 = (|Z|)^2/r_1 - r_1$$
$$\varphi_p = 2\beta + \varphi \text{ where } \tan\beta = (r_1 \sin\varphi)/(|Z| - r_1 \cos\varphi)$$
$$\text{Rad} = (r_2)/(2 \sin\varphi_p)$$

The semicircle obtained from the radius calculated in this way was always checked by inserting values of the impedance vector at regular intervals

The *rate of H secretion* into the mucosal solution was measured continuously by the pH stat method of Durbin and Heinz (1958) *i e* the pH on the mucosal side was kept constant at a predetermined level by infusion of a recorded volume of 15 meq/l sodium hydroxide solution (see Table I from an autoburette under automatic control from the glass calomel electrode of the mucosal side

TABLE I Composition (mM) of the ordinary mucosal and serosal side solutions and the solution for titration

Compound	Serosal	Mucosal	Titration
NaCl	81 [illegible]	P0 8	81 4
Na_2SO_4		10 8	1 5
KCl	3 2	4 0	4 0
$CaCl_2$	1 8	1 8	
$MgSO_4$	0 8	0 8	
KH_2PO_4	0 8		—
$NaHCO_3$	17 8		
Glucose	2 0		
Mannitol	—	12 8	7 9
NaOH	—		15 0

Solutions and chemicals The compositions of the ordinary serosal (pH 7 12) and mucosal side solutions and the solution for titration are given in Table I The compositions of solutions containing a weak acid differed from those given in Table I only regarding minor changes in the concentrations of sodium, sulphate, chloride and mannitol, made to avoid changes in osmolarity These solutions were always prepared at the pH to be used in an experiment and [illegible]

of high reliability, described by Obring (1948) was used

Results

Serosal side acetate as substrate for hydrogen ion secretion

Experiments were performed during 2 consecutive hours In the first hour the ordinary mucosal and serosal side solutions were used and no histamine was added The results obtained in this hour were used as controls In the second hour the serosal side solution contained 10 meq/l of acetate On both sides of the mucosa the pH was 7 12 Acetate alone had no significant effects on the hydrogen ion secretion or electric parameters in mucosae isolated from *Rana temporaria*

A second series of experiments was conducted during 3 consecutive hours, the results obtained in the first hour being used as controls During the second hour histamine was given and during the third hour both histamine and 10 meq/l of acetate The results are presented in Table II Histamine alone increased the hydrogen ion secretion rate and decreased the short-circuit current ($p<0\ 01$ for both) The *hydrogen ion secretion rate was higher on treatment with both histamine and acetate* than with histamine alone ($0\ 02 < p < 0\ 05$), but no significant change of the short-circuit current was noted Thus acetate increased the hydrogen ion secretion only in the presence of histamine As histamine was found necessary for the exertion of a stimulatory effect upon the hydrogen ion secretion by a weak acid, it was used throughout in all the subsequent experiments in this study

TABLE II Hydrogen ion secretion rate (H_r) active charge transport as short-circuit current (I_{sc}), transmucosal electric potential difference (U) and resistance (R) in a control h, an h with histamine alone and an h with histamine and 10 meq/l acetate on the serosal side The mean values for the last 30 min in each h ± S E are given n = 6

	H_r (μeq h^{-1}, cm^{-2})	I_{sc} (μeq h^{-1} cm^{-2})	U (mV)	R (ohm/cm²)
Control (1st exp hr)	0 54 ±0 13	1 69 ±0 30	25 8 ±2 6	532 ±44
Histamine (2nd exp hr)	1 75 ±0 27	1.20 ±0.22	21 4 ±1 7	679 ±90
Histamine and acetate (3rd exp hr)	2 39 ±0 25	1 27 ±0 18	20 1 ±1 3	571 ±60

Migration of undissociated weak acids and their dissociated anions

It is possible that migration of some weak acids into the cells of the isolated frog gastric mucosa results in an increase in the rate of hydrogen ion secretion The rate on addition of the unionized forms to the muscosal side solution would thus give an estimate of their migration into the cells

If the influx of an undissociated acid or its dissociated anion into the mucosal cells were greater than the efflux and/or metabolism this would mean a progressive increase in the intracellular concentration of the acid or its anion After cessation of the influx the intracellular concentration would decrease continuously The hydrogen ion secretion rate after the removal of a weak acid in the undissociated or the dissociated form from the mucosal side solution would thus indicate also whether an intracellular accumulation had taken place

In experiments performed over a period of several hours the hydrogen ion secretion of a mucosa washed in an oxygenated frog Ringer solution with addition of only glucose and histamine decreases quasiexponentially with time This seems most likely to be due to progressive mucosal exhaustion Consequently the basal hydrogen ion secretion declines continuously during an experiment This must always be borne in mind when comparing values at different times and will be referred to hereinafter as the decay curve

With a weak acid in the mucosal side solution it is possible that some of the hydrogen ions secreted are not available for titration At a low pH on the mucosal side there may be a loss of hydrogen ions from the solution due to evaporation of the dissociated volatile acids Back diffusion of hydrogen ions from the mucosal to the serosal side along the gradient of pH may also contribute to an apparent decrease in the rate of secretion

At a mucosal side pH of about 7 undissociated acid may be formed in the low pH environment in the immediate vicinity of the secretory cell Most if not all of this will however dissociate in the circulating neutral solution so that any evaporation loss in this way will be small

An approximate measure of the apparent decrease in hydrogen ion secretion in experiments with weak acids in the mucosal side solution was obtained from the two following types of control experiments

Diffusion loss of hydrogen ions

Teorell (1947) suggested back diffusion of hydrogen ions from the gastric lumen (mucosal side solution to be one important mechanism in reduction of the acidity of the gastric content Experimental support for this hypothesis has been given by several authors (*cf* Öbrink 1948 Terner 1948 Nordgren 1963 Lindner 1961 and Öbrink and Waller 1965

Experiments were performed during 3 consecutive hours The serosal side solution contained 10 meq/l of acetate as substrate and histamine was given throughout On the mucosal side the ordinary solution without addition of a weak acid was used pH in the 3 hrs being maintained at 7 12 4 00 and 7 12 respectively The results

TABLE III Effects of a change of pH from 7 12 to 4 00 and the reverse in the mucosal side solution without acid. The notation is the same as in Table II The mean values for the last 30 min in each h±S E are given n = 7

	H_r (μeq h^{-1}, cm^{-2})	I_{sc} (μeq h^{-1}, cm^{-2})	U (mV)	R (ohm cm^2)
pH = 7 12 (1st exp hr)	2 54 ±0 13	1 82 ±0 19	21 1 ±2 5	406 ±23
pH = 4 00 (2nd exp hr)	1 88 ±0 19	1 98 ±0 15	22 5 ±1 7	399 ±23
pH = 7 12 (3rd exp hr)	2 36 ±0 21	2 10 ±0 11	25 0 ±1 5	417 ±21

are given in Table III The measured rate of hydrogen ion secretion was significantly smaller ($p < 0\ 01$ for both) at a mucosal side pH of 4 00 than at a preceding or subsequent pH of 7 12 The values of the electric parameters obtained at a mucosal side pH of 4 00 were not significantly different from those obtained at pH 7 12 which probably indicate that the true rate of hydrogen ion secretion remained unchanged (*cf* Rehm 1962) although some of the hydrogen ions escaped by back diffusion

The result is in agreement with the hypothesis of Teorell The mean apparent reduction (0 66 μeq h^{-1}, cm^{-2}) is used (Table IV) to compensate for the diffusional escape of hydrogen ions in experiments where the pH in the acid containing mucosal side solutions was varied in the same way The compensation thus made is probably too small as hydrogen ions bound as undissociated acid migrate more rapidly in the gastric mucosa than unbound hydrogen ions (Flemström and Frenning 1968)

Evaporation loss of hydrogen ions

These measurements were performed in order to determine whether there was a loss of hydrogen ions from the circulating mucosal side solution *per se* when it contained a volatile weak acid in the undissociated or the dissociated form The reservoir, the connection tubes and the titration equipment on the mucosal side were used A glass tube was inserted between the connection tubes instead of the chamber compartment and the mucosa which were thus omitted Hydrogen ions were supplied to the solutions by continuous infusion of a 15 meq/l hydrochloric acid solution at a constant rate corresponding to a secretion rate of 3 μeq h^{-1} cm^{-2}

The rate of titration was recorded during three consecutive hours In the first hour the ordinary mucosal side solution was used pH kept at 7 12) In the second hour the solution contained 10 meq/l of dissociated acid (pH 7 12) and in the third hour 10 meq/l of acid partly in the undissociated form (pH 4 00) Acetate propionate, L-lactate and D lactate were used

When the mucosal side solution contained any of the acids an apparent decrease of the secretion rate was found both at a pH of 7 12 and at a pH of 4 00 The losses obtained in these experiments are used (Table IV to compensate for the corresponding losses in experiments on the isolated mucosa

TABLE IV The rate of hydrogen ion secretion (μeq, h^{-1}, cm^{-2}) in a control hour without acid and in an hour with 10 meq/l of dissociated (pH = 7 12) and undissociated (pH = 4 00) acid in the mucosal side solution. The loss of hydrogen ions due to evaporation (EV) of acid from the solutions and due to diffusion (DIF) along the gradient of pH are added to the rate of titration (TIT) to obtain the approximate rate of secretion (SEC) For all types of experiments the mean values for the last 30 min in each experimental hour ± S E are given With all the acids n = 6 for EV and n = 7 for DIF

		Acetate (n = 8 for TIT)	Propionate (n = 5 for TIT)	L-lactate (n = 7 for TIT)	D lactate (n = 7 for TIT)
Control pH = 7 12 (1st exp hr)	TIT = = SEC	2 28±0 25	2 17±0 13	3 25±0 42	3 13±0 18
Weak acid pH = 7 12 (2nd exp hr)	TIT	1 20±0 27	1 10±0 09	2 72±0 38	2 00±0 22
	+ EV	0 63±0 08	0 30±0 08	0 27±0 06	0 29±0 12
	= SEC	1 83±0 28	1 40±0 12	2 99±0 38	2 29±0 25
Weak acid pH = 4 00 (4th exp hr)	TIT	2 03±0 25	1 11±0 36	2 41±0 17	1 14±0 21
	+ EV	1 33±0 13	1 26±0 13	1 36±0 30	1 19±0 11
	+ DIF	0 66±0 14	0 66±0 14	0 66±0 14	0 66±0 14
	= SEC	4 02±0 31	3 03±0 41	4 43±0 37	2 99 0 28

Influx of weak acids into the mucosal cells

Experiments with acetate (pK_a = 4 78), propionate (pK_a = 4 86), L-lactate (pK_a = 3 90) and D-lactate (pK_a = 3 90) were performed during 5 consecutive hours In the first the third and the fifth hour the mucosal side solution contained no acid and the pH was kept at 7 12 The results obtained in these hours were used as controls In the second hour the mucosal side solution contained 10 meq/l of a weak acid The pH was kept at 7 12 and most of the acid was thus in the dissociated form In the fourth hour the same concentration of acid was used, but the pH was kept at 4 00 which meant that a considerable proportion of all the acids used was in the undissociated form No acids were added on the serosal side, where the pH was 7 12 Histamine was given throughout the experiments

After approximate correction for the apparent decrease in the rates of secretion due to evaporation of volatile acid and back diffusion of hydrogen ions the rate of hydrogen ion secretion was higher with acetate, propionate and L-lactate at a pH of 4 00 than in the preding control hour without acid (Table IV) Conversely, it was lower with D-lactate as might be expected from the "decay curve" if D-lactate did not stimulate These results strongly suggest that weak acids migrate rapidly into the acid secreting cells when in the undissociated form D lactate did not stimulate hydrogen ion secretion but as it seems plausible to assume that the intracellular migration of D-lactate is about the same as that of L-lactate the increased hydrogen ion secretion is presumably due to a substrate effect of the other acid anions

With all acids, at pH 7 12 the calculated secretion rate was lower than that in the control hour It should be stressed however that this decreased secretory rate

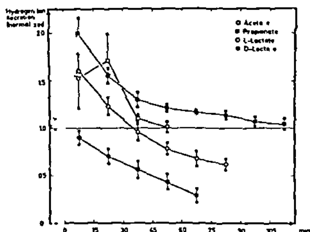


Fig 4 The mean rates of hydrogen ion secretion ±S E after removal of the *undissociated* acids (10 meq/l pH – 4 00) from the mucosal side solution Zero time indicates the time of change from an acid containing (dashed lines) to the ordinary (solid lines) mucosal side solution The data were normalized to reduce the influence of individual mucosal variation For each mucosa the rates of secretion after treatment with acid were divided by that in a control period before treatment with acid (the last 30 min in the first experimental hour) The line y = 1 0 indicates the case of an unchanged rate of acid secretion. One half of the ±S E lines was omitted for some points

may reflect the decay curve and some back diffusion of hydrogen ions bound as undissociated acid formed in the vicinity of the secretory cells For this reason a small influx of dissociated anions or formed undissociated acids cannot be excluded At pH 7 12 however the rate of hydrogen ion secretion due to an influx is much smaller than with a pH 4 00 *even though in the latter experiments the decrease due* to the decay curve of hydrogen ion secretion is greater

In order to determine whether intracellular accumulation of an undissociated weak acid had occurred the rate of hydrogen ion secretion in a control period after treatment with an acid was compared with the rate before treatment with an acid In Fig 4 (undissociated acids) and Fig 5 (dissociated acids) the rate after addition of acid was compared with the mean rate during the last 30 min in the first control hour (*i e* the first experimental hour) The data were normalized to reduce the influence of individual mucosal variation

After removal of the undissociated acid from the muscosal side solution there was an increase in the rate of hydrogen ion secretion presumably indicating that intracellular accumulation of acetate propionate and L lactate had occurred After treatment with acetate the hydrogen ion secretion remained significantly increased during two consecutive 15 min periods ($0 01 < p < 0 02$) and ($0 02 < p < 0 05$) After propionate it was increased during four 15 min periods ($0 02 < p < 0 05$), ($0 02 < p < 0 05$) ($p < 0 01$) and ($p < 0 01$) After L-lactate is was increased during two 15 min periods ($p < 0 01$) and ($0 01 < p < 0 02$), respectively The results with undissociated acids were, in principle the same when compared with the mean rates during the last 30 min in the control period immediately preceding acid treatment (*i e* the third experimental hour)

After treatment with undissociated acetate, propionate and L-lactate the individual variations in hydrogen ion secretion were considerable in the first and second 15 min periods This was partly due to the fact that maximal secretion in some ex

TABLE IV [illegible]

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Weak acid pH = 7 12 (2nd exp hr)	TIT	1 20 ± 0 27	1 10 ± 0 09	2 72 ± 0 38	2 00 ± 0 22
	+ LV	0 63 ± 0 08	0 30 ± 0 08	0 27 ± 0 06	0 29 + 0 12
	= SEC	1 83 ± 0 28	1 40 ± 0 12	2 99 ± 0 38	2 29 ± 0 25
Weak acid pH = 4 00 (4th exp hr)	TIT	2 03 ± 0 25	1 11 ± 0 36	2 41 ± 0 17	1 14 + 0 21
	+ LV	1 33 ± 0 13	1 26 ± 0 13	1 36 ± 0 30	1 19 ± 0 11
	+ DIF	0 66 ± 0 14	0 66 ± 0 14	0 66 ± 0 14	0 66 + 0 14
	= SEC	4 02 + 0 31	3 03 + 0 41	4 43 ± 0 37	2 99 0 28

Influx of weak acids into the mucosal cells

Experiments with acetate (pK_a = 4 78), propionate (pK_a = 4 86), L-lactate (pK_a = 3 90) and D lactate (pK_a = 3 90) were performed during 5 consecutive hours In the first the third and the fifth hour the mucosal side solution contained no acid and the pH was kept at 7 12 The results obtained in these hours were used as controls In the second hour the mucosal side solution contained 10 meq/l of a weak acid The pH was kept at 7 12 and most of the acid was thus in the dissociated form In the fourth hour the same concentration of acid was used but the pH was kept at 4 00 which meant that a considerable proportion of all the acids used was in the undissociated form No acids were added on the serosal side where the pH was 7 12 Histamine was given throughout the experiments

After approximate correction for the apparent decrease in the rates of secretion due to evaporation of volatile acid and back diffusion of hydrogen ions the rate of hydrogen ion secretion was higher with acetate propionate and L-lactate at a pH of 4 00 than in the preding control hour without acid (Table IV) Conversely, it was lower with D lactate as might be expected from the 'decay curve' if D-lactate did not stimulate These results strongly suggest that weak acids migrate rapidly into the acid secreting cells when in the undissociated form D lactate did not stimulate hydrogen ion secretion but as it seems plausible to assume that the intracellular migration of D lactate is about the same as that of L-lactate the increased hydrogen ion secretion is presumably due to a substrate effect of the other acid anions

With all acids, at pH 7 12 the calculated secretion rate was lower than that in the control hour It should be stressed however that this decreased secretory rate

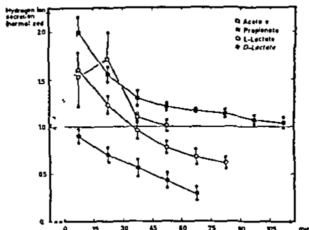


Fig 4 The mean rates of hydrogen ion secretion ±S E after removal of the *undissociated* acids (10 meq/l pH = 4 00) from the mucosal side solution Zero time indicates the time of change from an acid containing (dashed lines) to the ordinary (solid lines) mucosal side solution The data were normalized to reduce the influence of individual mucosal variation For each mucosa the rates of secretion after treatment with acid were divided by that in a control period before treatment with acid (the last 30 min in the first experimental hour) The line y = 1 0 indicates the case of an unchanged rate of acid secretion One half of the ±S E lines was omitted for some points

may reflect the "decay curve' and some back diffusion of hydrogen ions bound as undissociated acid formed in the vicinity of the secretory cells For this reason a small influx of dissociated anions or formed undissociated acids cannot be excluded At pH 7 12, however, the rate of hydrogen ion secretion due to an influx is much smaller than with a pH 4 00 even though in the latter experiments the decrease due to the 'decay curve' of hydrogen ion secretion is greater

In order to determine whether intracellular accumulation of an undissociated weak acid had occurred, the rate of hydrogen ion secretion in a control period after treatment with an acid was compared with the rate before treatment with an acid In Fig 4 (undissociated acids) and Fig 5 (dissociated acids) the rate after addition of acid was compared with the mean rate during the last 30 min in the first control hour (*i e* the first experimental hour) The data were normalized to reduce the influence of individual mucosal variation

After removal of the undissociated acid from the muscosal side solution, there was an increase in the rate of hydrogen ion secretion presumably indicating that intracellular accumulation of acetate propionate and L lactate had occurred After treatment with acetate the hydrogen ion secretion remained significantly increased during two consecutive 15 min periods (0 01 < p < 0 02) and (0 02 < p < 0 05) After propionate it was increased during four 15 min periods (0 02 < p < 0 05), (0 02 < p < 0 05), (p < 0 01) and (p < 0 01) After L lactate is was increased during two 15 min periods (p < 0 01) and (0 01 < p < 0 02), respectively The results with undissociated acids were in principle the same when compared with the mean rates during the last 30 min in the control period immediately preceding acid treatment (*i e* the third experimental hour)

*After treatment with undissociated acetate propionate and L-lactate the indi*vidual variations in hydrogen ion secretion were considerable in the first and second 15 min periods This was partly due to the fact that maximal secretion in some ex

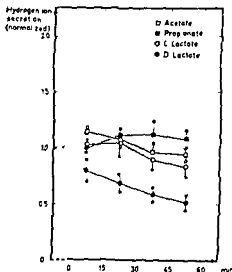


Fig 5

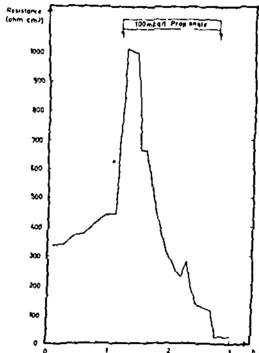


Fig 6

Fig 5 The mean rates of hydrogen ion secretion ±S E after removal of the *dissociated* acids 10 meq/l pH = 7 12) from the mucosal side solution The symbols and the normalization re the same as in Fig 4

Fig 6 Changes of the ohmic resistance when the mucosal side solution without a weak acid s changed for a mucosal side solution with 100 meq/l of propionate *Most of the propionate* as in the undissociated form (pH = 4 00)

periments occured in the second 15 min period, the secretion in the first period after treatment with acid being lower than in the period before acid treatment The variations may indicate that a large amount of acid which had accumulated intracellularly transiently inhibited acid secretion in some of the mucosae

In contrast to the result with these the other acids a decrease was found in the secretion rate following treatment with D-lactate at pH 4 00 The decrease was significant *from the beginning of the third 15 min period* $(0\,02 < p < 0\,05)$ *and* onwards Assuming the same mechanism of migration of the L- and D isomers of lactic acid the result with D-lactic acid makes it less likely that the increase in secretion on change from a low to a high pH value could be due to the release of undissociated acid trapped in mucous layers or other sites at the cell surfaces

With none of the acids used here was there a significant change in the hydrogen ion secretion during the control hour after a period with dissociated acid (pH 7 12) in the mucosal side solution There was a difference, however between the individual acids in that the secretion rate remained unchanged after treatment with acetate,

propionate and L lactate but decreased slowly after D lactate (*cf* Fig 5) If the decreasing values with D lactate reflect decay of the basal secretion, then other acids produce an increase in serection A small flux into the muscosal cells of the dissociated anions or undissociated acid formed in the vicinity of the cells thus can be assumed to have taken place

The magnitudes of the electric parameters in these experiments were, in principle, the same as those given in Table III With acetate at a pH of 4 00 the electric potential difference was smaller than in the control periods ($0\,02 < p < 0\,05$) With propionate the resistance was greater at a pH of 4 00 ($0\,02 < p < 0\,05$) than at a pH of 7 12 With D lactate the resistance and the electric potential difference increased successively The increase was significant from the second experimental hour for the resistance and from the fourth experimental hour for the electric potential difference ($0\,02 < p < 0\,05$ for both) No other significant differences in the electric parameters in experiments with one acid were found

Intracellular accumulation and permeability effects

Acids which have been shown earlier to increase the permeability to ions when instilled into the gastric lumen in experiments with cats and dogs *in vivo* apparently decreased the rate of hydrogen ion secretion (Davenport 1967) This is contrary to the results obtained above that these acids increased the secretion in the frog gastric mucosa when present in an undissociated from on the mucosal side

As the concentrations used in the mammalian experiments were much higher than that used here, this may account for the different effects The effect of a high con centration of a weak acid on the ionic permeability in the gastric mucosa was therefore studied

A mucosal solution with a pH of 4 00 containing 100 meq/l of propionate was used after a control period with the ordinary mucosal side solution at a pH of 7 12 Resistance and impedance locus diagram determinations were used to obtain a measure of the mucosal permeability to ions Fig 6 and 7 show 2 typical experiments With 100 meq/l or propionate, the parallel resistance determined from the impedance locus diagram and the resistance determined with direct current first rose to a maximum which was then followed by a large decrease It could thus be con cluded that 100 meq/l of propionate after a transient decrease, gave rise to an n crease in the permeability to ions in the frog gastric mucosa when present mainly in an unionized form on the mucosal side In principle the same effect was obtained with 100 meq/l of acetate

Only minor changes in the polarization angle (φ_p) were found This might in dicate unchanged polarization capacity in the mucosa No measurement of hydrogen ion secretion was performed with 100 meq/l of acid in the mucosal side solution The short circuit current and the electric potential difference decreased to low values

In some experiments the period with 100 meq/l of undissociated acid was fol by a control hour Du[illegible]

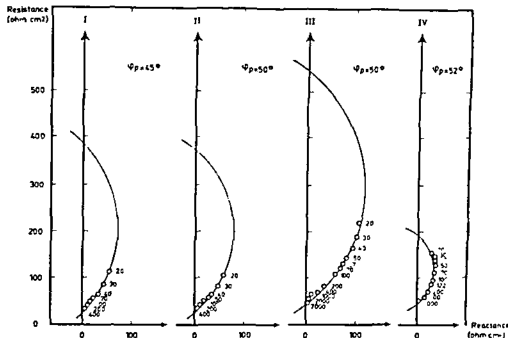


Fig 7 Impedance locus diagram in 1 hour with the non acid containing mucosal side solution and in the following hour when a mucosal side solution with 100 meq/l of propionate was used Most of the propionate was in the undissociated form (pH = 4 00) Diagram I was obtained 15 min and diagram II 45 min after the start of the experimental hour without acid Diagrams III and IV were obtained 15 and 45 min after change to the mucosal side solution with propionate

smaller than before the treatment with acid The electric potential difference and the resistance increased slowly and the short circuit current more rapidly The magnitudes of the electric parameters were, however, considerably smaller after than before the treatment with acid

Discussion

Acetate and propionate as substrates for hydrogen ion secretion

Acetate given on the serosal side increased the hydrogen ion secretion in mucosae from *Rana temporaria* only in the presence of histamine This result is in conflict with that of Alonso *et al* 1967 who found that acetate alone increased the secretion in mucosae from the bullfrog (*Rana catesbeiana*) These divergent results may be due to the difference in species Both acetate and propionate mainly in an unionized form in the mucosal side solution increased secretion These experiments were only performed with simultaneous histamine stimulation

As previously shown by Alonso *et al* (1967 there was no reduction in the short-circuit current when the hydrogen ion secretion was stimulated by a short-chain fatty acid Assuming th the short-circuit current represents the difference in tran

sport velocity between chloride and hydrogen ions, this might indicate a contribution of fatty acids to some metabolic machinery common to hydrogen and chloride ion transport mechanisms

L-lactate and D lactate as substrate for hydrogen ion secretion

From the effect on the hydrogen ion secretion it was concluded that lactate was a metabolic substrate in mucosae isolated from *Rana temporaria*

A stimulatory effect of DL-lactate upon the secretion was obtained by Davenport and Jensen Chavre (1950) in the mouse stomach *in vitro* In these experiments the solutions contained 1 mg % of carbaminoyl choline In conflict with the present results and with those of Davenport and Chavre Alonso *et al* (1967) found that lactate had no stimulatory effect on the secretion of hydrogen ions in isolated mucosae from *Rana catesbeiana* They ascribed the absence of an increase in secretion to limited availability of a lipoic cofactor necessary for the conversion of pyruvate to acetyl CoA by keto acid dehydrogenase They did not use histamine in their experiments however and it is possible that histamine or a cholinergic substance may be necessary for stimulation of hydrogen ion secretion by L-lactic acid This difference in the results might also be due to the difference in species or it could be due to the regular feeding of the frogs in the present study with liver

Migration of weak acids in the gastric mucosa

By using the substrate effect of acetate propionate and L lactate it was shown that these acids migrated rapidly into the acid secreting cells when the pH on the mucosal side was 4 00 At this pH value these acids are unionized to a considerable degree When the pH on the mucosal side was 7 12 and the acids were thus almost completely dissociated the influx appeared to be much slower

An accumulation of weak acids in the mucosal cells on migration from the gastric lumen can be assumed to take place if the influx is greater than the disappearance With weak acids which can serve as substrates for hydrogen ion secretion both metabolism and diffusion would contribute to the disappearance The persistence of the stimulation of secretion after removal of the acids from the mucosal side fluid strongly suggests that they accumulate in the acid secreting cells The raised secretion values persisted for a period of 30 min after treatment with acetate and L-lactate and for 60 min after treatment with propionate

Hogben (1954 1962) considered that both dissociated DL-lactate and DL-lactic acid permeated the frog gastric mucosa *in vitro* as undissociated acid With the mucosal side pH lower than that on the serosal side he found that the flux from the mucosal to the serosal side was much greater than that in the opposite direction The flux ratio was the same with the lower pH on the serosal side when the mucosae were spontaneously secreting hydrogen ions

The disappearance rate for acetate was greater on installation of acetic acid than sodium acetate in the cat stomach (Teorell 1939 Flemstrom and Frenning 1968) It was however considerable for both the ionized and the unionized species

Both the dissociated and the undissociated species of a weak acid thus appear to migrate through the mucosa. The flux of an acid into the cells, however, takes place more rapidly when the acid is in the undissociated form. This means that the cell membranes are more permeable to the unionized acid than to its ionized anion. If the unionized acid which has entered the cells is ionized at the intracellular pH, then the efflux from the cell will consist of anions and will thus be slow. Under these conditions when unionized acids enter the cells there will be a tendency for the anions to accumulate in the cells.

The rate of migration of an undissociated weak acid through the whole mucosa would reflect the permeability of the liberated anion on the serosal side of the cells and the intracellular concentration of the anion. Under steady state conditions the rate of migration would be greater for the undissociated than for the dissociated species of a weak acid, as the intracellular concentration of the acid anions entering the cells as undissociated acid would be higher.

While it does not appear necessary to postulate different pathways of migration for the two species, it might be possible that the pathways is transcellular for the undissociated acid but in the main intercellular for the anion.

Accumulation of weak acids in the cells should result in intracellular liberation of hydrogen ions and anions. The former would cause a reduction of the intracellular pH, i.e. an acidosis, and the increased intracellular ionic content would result in hypertonicity and mucosal swelling. The impedance locus diagram corresponded well with the semicircular form also when the permeability had increased. With metabolic acidosis due to a reduction of the oxygen supply more complex impedance loci were obtained (Flemstrom 1971). This difference in locus diagrams might be explained as due to an increase of the intracellular ionic content in experiments with undissociated acids.

Weak acids and gastric mucosal permeability to ions

At a concentration of 100 meq/l undissociated propionic or acetic acid which apparently accumulates in the acid secreting cells increased the ion permeability of the frog gastric mucosa. It is therefore not improbable that the mechanism underlying the permeability increase was the intracellular accumulation of the acid.

As estimated from the resistance and the locus diagram determinations there was first a transient decrease in the ionic permeability followed by an increase. The transient decrease might be explained by a low grade of hypertonic cellular swelling reducing the extracellular space and thus possibly one pathway for the migration of ions. The subsequent increase might then be due to an increase of the extracellular space between mucosal cells which would acquire a spherical shape when more swollen.

The weak acids increased the ionic permeability of the frog gastric mucosa at the same concentration as that found by Davenport (1964) to increase the ionic permeability on instillation into the dog stomach. The permeability increasing effect of undissociated weak acids obtained in experiments on mammalian stomachs in vivo

seems, however, to appear more rapidly than the increase observed in experiments on the frog gastric mucosa *in vitro* It is therefore possible that an increase in the blood flow (*cf* Augur 1970) due to local acidosis contributes to the permeability increase in experiments with intact stomachs Intracellular liberation of hydrogen ions from the accumulating weak acid probably results in a local acidosis

Flemstrom and Frenning (1968) conducted to experiments with repeated instillations of hydrochloric acid into the cat stomach before and after a 30 min instillation of 170 meq/l acetic acid An increased permeability for ions that persisted for 90 min was found The accumulated weak acids apparently remained in the frog gastric mucosal cells for some time after the change from an acid to a non acid containing solution on the mucosal side It is thus possible that intracellular accumulation of undissociated weak acids can account for their transiently persistent effect on the mucosal permeability to ions in the intact mammalian gastric mucosa

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From the Department of Pharmacology, Karolinska Institutet Stockholm Sweden

Dissociation of Tracer Disappearance Rate and Blood Flow in Isolated Skeletal Muscle during Various Vascular Reactions

By

PER BOLME and LENNART EDWALL

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Abstract

BOLME, P and L EDWALL *Dissociation of tracer disappearance rate and blood flow in isolated skeletal muscle during various vascular reactions* Acta physiol scand 1971 *82* 17—27

The rate of disappearance of diffusible radioactive tracers (xenon and iodide) and blood flow were measured in the
ratio between disappe
resulted in TFI values
arterial infusion of ac
transport of tracer bet
blood flow by low frequency stimulation of vasoconstrictor nerves or by i a. infusion of nor adrenaline resulted in TFI s approaching 1, indicating an approximately equal effect on tracer transport and blood flow However, high frequency stimulation of vasoconstrictor nerves reduced tracer transport more than blood flow During partial occlusion of the arterial inflow the iodide TFI was greater than 1, suggesting a compensatory increase in tracer transport relative to blood flow The present results indicate that the disappearance rates of xenon and iodide are not only dependent on total blood flow in the muscle but are also influenced by local factors in the capillary section affecting the exchange function Thus the calculation of muscle blood flow from xenon disappearance data alone may give misleading results

Kety (1949) suggested that the clearance rate of a locally injected diffusible water soluble tracer could be used as a quantitative measure of the local circulation It was pointed out that the clearance of such a tracer, although mainly blood flow dependent, could be augmented by opening the capillaries or by increased filtration This has been experimentally confirmed by the findings that the functional capillary surface area and the fluid filtration influence the rate of transcapillary transport of water soluble tracers (Renkin and Rosell 1962 a, Renkin, Hudlicka and Sheehan 1966, Lundgren and Mellander 1967)

Thus both total blood flow and local adjustments in the capillary section of a vascular bed may affect the rate of disappearance from a locally injected tracer depot In a previous study this possibility was made use of by combining meas

ments of total blood flow with tracer disappearance studies (Bolme and Edwall 1970) With this procedure it was possible to dissociate vascular responses in skeletal muscle caused by activation of cholinergic sympathetic vasodilator fibres from those due to motor nerve stimulation

The aim of the present study was to see whether there is a dissociation between tracer disappearance rate and blood flow during other types of vascular reaction, namely metabolic vasodilatation, sympathetic vasoconstriction and the influence of vasoactive drugs

Methods

[illegible] e anesthetized [illegible] mius plantaris [illegible] l as *M flexor* [illegible] s the muscles [illegible] ip recorder in [illegible] Sweden) In [illegible] ase (4 expts) *the flowmeter measured the blood flow to the muscle by a probe fitted on the femoral artery* Pressure in the cannulated common carotid artery was measured by a Statham pressure transducer (P 23 AC) The recordings were made on a Grass model 5 Polygraph A branch of *A poplitea* was cannulated and used for infusions The prepared limb was immobilized by screws in the tibia and femur Rectal temperature was continuously monitored and the body [illegible] ting lamps The isolated muscle was kept [illegible]

[illegible] rical stimulation of the cut tibial nerve [illegible] ty 4 V frequency 1—10 imp/sec) The stimuli were delivered by a Grass model S4G stimulator

2 Graded vasodilatation by infusion of acetylcholine (1 μg/ml i a) Three methods for reduction of blood flow were employed 1 Partial occlusion of the femoral artery 2 Direct electrical stimulation of the cut lumbar sympathetic chain 3 Infusion of noradrenaline (1 μg/ml i a The sympathetic chain reached by a retroperitoneal approach was stimulated with a bipolar silver electrode at the level of L4—L5 (0 1—2 0 msec 4—8 V 0 25—10 imp/sec) *The different maneuvres to control blood flow were compared in the same animal and the* order in which they were instituted was randomized

Xe^{133} in isotonic saline solution was obtained with activities of 20—150 mCi/ml I^{125} and I^{131} as iodide stabilized with sodium thiosulphate and dissolved in isotonic phosphate buffer pH 7—8 were obtained with activities of 20—40 mCi/ml (AB Atomenergi Studsvik Nyköping Sweden Radiochemical purity was checked by the suppliers by means of gamma spectrometry I^{125} was injected alone and Xe^{133} either alone or mixed with I^{131} The tracer solutions were injected intramuscularly 0 010 -0 40 ml) during 1 5—4 0 min The tracer depot was monitored externally by a scintillation detector connected to a preamplifier and two single channel pulse height analyzers each fed into two scalers For further technical details see Bolme and Edwall 1970

The experiments were started 7 15 min after the isotope injection when the isotope disappearance rate was predominantly monoexponential This phase usually lasted for [illegible]—40 min Radioactivity was counted for periods of 12 24 or 60 sec Three to four subsequent isotope injections could usually be made in the same animal The physical background was determined before the experiments Furthermore in the double tracer expriments the pulse rates in both *recording channels were determined separately for each of the isotopes* 14 or 15 % of the gross pulse rate recorded in the I^{131} channel was registered in the Xe^{133} channel and this was corrected for in all double tracer experiments Corrections for total final background and determinations of disappearance rate k) were performed as described earlier (Bolme and Edwall 1970

In order to compare the responses during different vascular reactions a transport flow index (TFI) has been defined as the ratio between the disappearance rate and the blood flow both expressed as percent of control

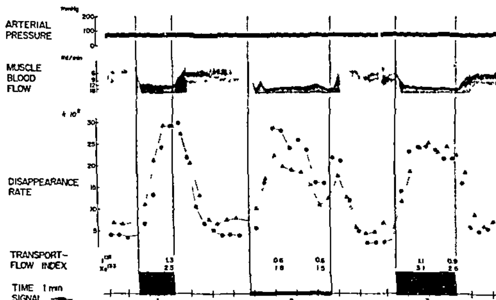


Fig 1 Dog 11 kg Pentobarbital *M gastrocnemius* 54 g Comparison between vasodilatations

1 Motor nerve stimulation 4 V, 0 01 msec, 3 imp/sec
2 Acetylcholine infusion 0 3 μg/min i a
3 Motor nerve stimulation 4 V, 0 01 msec, 4 imp/sec
The disappearance rate is plotted as k 10^2/min according to Bolme and Edwall (1970). Isotopes I^{131} ▲, Xe^{133} ●

Results

Increase in blood flow

a) *Comparison between the effects of motor nerve stimulation and acetylcholine infusion*

The motor nerve was stimulated in 14 dogs and the vascular responses compared with those produced by acetylcholine infusion In 3 dogs the disappearance of Xe^{133} and I^{131} was simultaneously recorded (Fig 1) The resting k-value for Xe^{133} was about half the resting k-value of I^{131} The metabolic vasodilatation (Fig 1 1) produced about the same maximal disappearance rates for both tracers The maximal xenon disappearance rate was 720 % of control and the corresponding blood flow was 290 % of control giving TFI of 2 5 At the same time the iodide TFI was 1 3 Following acetylcholine infusion (Fig 1 2) the initial TFI for xenon was 1 8 and for iodide 0 6 If the xenon TFI in the late phase of the motor nerve stimulation (Fig 1 3) was compared with the TFI in the corresponding phase of the acetylcholine infusion (Fig 1 2) values of 2 6 and 1 5 respectively were obtained The corresponding iodide TFI values were 0 9 and 0 6 respectively The data in Fig 1 were obtained 7—24 min after the end of the isotope injection At longer times after injection the control k-values were generally lower and the TFI values during metabolic

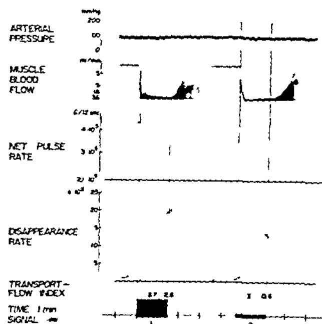


Fig 2 Dog 11 kg Pentobarbital. M gastrocnemius 53 g Comparison between vasodilatations in the late part of a disappearance run Isotope Xe^{133}
1 Motor nerve stimulation 4 V, 0 01 msec 2 imp/sec
2 Acetylcholine infusion 1 μg/min i a

TABLE I Initial effects of vasodilation produced by acetylcholine infusion and motor nerve stimulation expressed as means of TFI values (Means were calculated using logarithms of the TFI values

	Acetylcholine		*Motor nerve stim*	
Dog no	Xenon	Iodide	Xenon	Iodide
1	0 96	0 49	2 48	1 45
2	0 97	0 89	2 65	1 63
3	2 00	1 77	1 52	1 86
4	1 67		1 35	
5		0 68		1 64
6	1 52	0 47	3 62	1 00
7	1 30		4 03	
8		2 54		5 74
9		2 86		3 40
10	2 26	2 13	5 85	4 75
11	1 09		1 6[illegible]	
12	1 83	0 60	2 77	1 22
13	1 4[illegible]	0 52	1 53	0 86
14	2 [illegible]	0 89	1 78	0 99
Variance within dogs*	1 12	0 62	0 41	0 52

* The mean square within dogs

vasodilatation were somewhat higher in comparison with the TFI values following acetylcholine infusion One such experiment is illustrated in Fig 2 where the metabolic vasodilatation Fig 2 1 was started 24 min after the radioactive xenon injection During this metabolic vasodilatation the TFI s were 3 7 and 2 6 The cor-

TABLE II Late effects of vasodilatations produced by acetylcholine infusion and motor nerve stimulation expressed as individual TFI values

Dog no	*Acetylcholine*		*Motor nerve stim*	
	Xenon	Iodide	Xenon	Iodide
1	0.40	0.70	1.95	1.45
	0.59		2.60	
	0.59			
2	0.64	0.65	2.36	1.81
3	1.80	1.44	1.62	1.74
	1.12		1.49	
	1.09			
4	0.80		2.01	
6	0.16	0.05	2.10	0.81
7	1.84		4.12	
9		0.46		2.88
				2.40
12	1.54	0.58	2.59	0.88
13	0.74	0.49	2.06	0.79
14	0.96	0.57	1.80	1.31

responding data during the subsequent acetylcholine infusion were considerably lower, 1.3 and 0.6

The mean TFI values from the first minute of vasodilatation are collected in Table I The intraindividual differences in TFI s during motor nerve stimulation and acetylcholine infusion were calculated for each tracer The mean difference ± S E in xenon TFI was 1.05 ± 0.43 which was almost significant ($p < 0.05$) For iodide this difference ± S E was 0.97 ± 0.30 which was significant ($p < 0.01$) It is conceivable that the high initial peak k values are to some extent due to wash out of tracer pooled in the blood (Lassen 1964) This would however, only affect the TFI s from the first minute of vasodilatation and would not affect TFI s obtained later during the vasodilatation

Therefore determinations were also performed in the late phase *i e* more than 1 min after the start of the vasodilatation in experiments where vasodilatation exceeded 1 min duration These results are collected in Table II The intraindividual differences between the two types of vasodilatation were calculated for each tracer Repeated observations were compared with respect to their means For xenon TFI s the mean difference ± S E was 1.37 ± 0.21 ($p < 0.001$) and for iodide 0.81 ± 0.22 ($p < 0.01$)

b) *Metabolic vasodilatation during restricted blood flow*

In order to induce maximal metabolic vasodilatation the motor nerve was stimulated during partial occlusion of the femoral artery *Single tracer measurements* (3 expts) as well as double tracer measurements (3 expts) were performed A double tracer experiment is illustrated in Fig 3 Blood flow was steady at 135 % of control about 2 min after the start of the stimulation At the same time the disappearance rate of xenon was 370 % and that of iodide 300 % of control The TFI s were

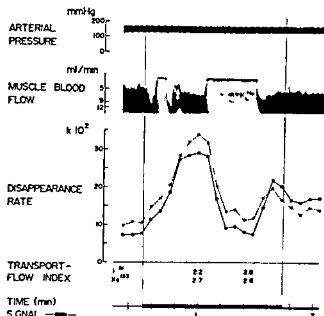


Fig 3 Dog 14 kg Pentobarbital *M gastrocnemius* 57 g Effect of metabolic vasodilatation during partial occlusion

1 Motor nerve stimulation 8 V, 0 01 msec 2 imp/sec during partial occlusion
2 Partial occlusion

Disappearance rate of I^{131}O and Xe^{133}□

and 2 2, respectively About 2 min later the blood flow level was less than 50 % of control whereas the isotope disappearance rates were still somewhat higher than control The TFI s for xenon and iodide were 2 6 and 2 8 respectively High TFI values during metabolic vasodilatation and restricted blood flow was a consistent observation in all experiments with both xenon and iodide For xenon the TFI varied between 1 96—4 05, and for iodide between 1 41 —2 80

c) Comparison between the effects

The cumulated data on the effects of increased blood flow on TFI are summarized in Fig 4 Motor nerve stimulation accompanied by partial occlusion resulted in TFI values of about 2 0—3 0 irrespective of whether measured during initial or late phases This was the case for both xenon and iodide TFI values of similar magnitude were obtained during initial phases of motor nerve stimulation without occlusion During the late phase of motor nerve stimulation the TFI values seemed to be reduced The TFI s during the late phase of acetylcholine infusion were even smaller iodide TFI (mean ± S E) was 0 62 ± 0 14 i e lower than 1 ($p<0.05$

Reduction in blood flow

Stimulation of the cut lumbar sympathetic trunk was performed in 10 expts (8 single tracer and 2 double tracer expts) Fig 5 illustrates a typical experiment in which the disappearance rate of I^{125} was measured Stimulation at 1 imp/sec produced comparable decreases in disappearance rate and blood flow giving a TFI of 1 1 while 6 imp/sec resulted in a TFI of 0 5

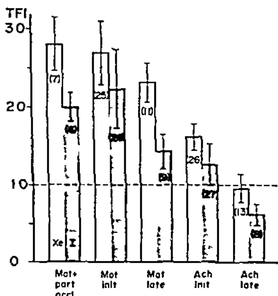


Fig 4 Effects of different types of increases in blood flow on the TFI values (mean±S E.) of Xe[133] (white bars) and of I[125] or I[131] (grey bars) Motor nerve stimulation with concomitant partial occlusion (Mot+part occl) Motor nerve stimulation first min (Mot init) motor nerve stimulation after 1 min (Mot late), acetylcholine infusion first min (Ach init) and acetylcholine infusion after 1 min (Ach late) Figures in the bars number of observations

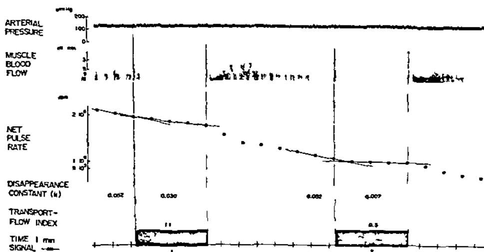


Fig 5 Dog 11 5 kg Pentobarbital *M gastrocnemius* 40 g Effect of direct stimulation of sympathetic vasoconstrictor nerves on total blood flow and disappearance rate of I[125] Sympathetic chain stimulation 8 V 1 msec

1 1 imp/sec
2 6 imp/sec

Disappearance rate is illustrated both as semilog plot of the net pulse rate and as k values

As can be seen from Fig 6 stimulation of the sympathetic vasoconstrictor nerves at higher frequencies (2 5—10 imp/sec) resulted in TFI values significantly lower than 1 for both xenon and iodide the mean TFI ± S E for xenon was 0 70 ± ($p<0.02$) and for iodide 0 63 ± 0 03 ($p<0.01$) At frequencies between 0

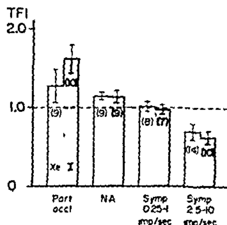


Fig 6 Effects of different types of reduction in blood flow on the TFI values (mean ±S E) of Xe^{133} (white bars) and of I^{125} or I^{131} (grey bars) Partial occlusion (part occl), noradrenaline infusion (NA), vasoconstrictor nerve stimulation (Symp) Figures in the bars number of observations

1 imp/sec, the TFI values were around 1 for both tracers In the majority of the experiments with sympathetic nerve stimulation the TFI values remained constant throughout the stimulation

Following noradrenaline infusion the TFI values were close to 1 and no difference could be observed between the tracers whether measured initially or late during the infusion In contrast during partial occlusion of the arterial inflow the iodide TFI values were higher than 1 (1 62 + 0 18 $p < 0.05$) For xenon a similar tendency was observed, although the changes were not significant These data were calculated during the first 3 min of occlusion During the latter period of partial occlusion the TFI values increased in contrast to responses during the other types of blood flow reduction

Discussion

The present results clearly demonstrate that changes in disappearance rates of both tracers upon a given change in total blood flow were dependent on the type of vascular reaction Thus although the volume flow of blood is an important factor determining the disappearance rate other factors are of quantitative importance for the disappearance rate of both iodide and xenon Such factors include changes in capillary permeability capillary exchange surface area and capillary flow distribution and velocity (cf Mellander and Johansson 1968)

It has been found that capillary permeability does not change during muscular exercise (Arturson and Kjellmer 1964 or during hypoxemia Scott Daugherty and Haddy 1967 and it therefore seems unlikely that it would be changed in the present experiments

It is well established that during metabolic vasodilatation the number of patent capillaries is increased Krogh 1919 and as a consequence, there is a larger exchange surface area This results in an increased net fluid filtration (Cobbold et al 1963, Kjellmer 1964) . d augmented transcapillary transport (Renkin and Rosell

1962 b, Renkin, Hudlicka and Sheehan 1966, Lundgren and Mellander 1967) Strong evidence has been presented that accumulation of local metabolites causes the profound vascular effects in exercising muscle (*cf* Mellander and Johansson 1968) On the other hand during increased blood flow which is not connected with elevated production of local metabolites, as may be the case during acetylcholine infusion, the capillary section may be less affected than during metabolic vasodilatation This may explain why TFI was less during acetylcholine infusion than during metabolic vasodilatation. Furthermore, high doses of acetylcholine infused i a may constrict arteriolar or precapillary elements, thus reducing the number of capillaries being perfused (Sonnenschein, Wright and Mellander 1967) Such an effect would tend to decrease the TFI

In addition, changes in blood flow distribution and velocity in patent capillaries may occur during i a infusion of acetylcholine, when the vascular resistance is reduced in the precapillary sphincter section In such situations blood flow may be diverted from long capillaries with a high "permeability surface area" (PS) product to short capillaries with a low PS (Sheehan and Renkin 1965, Renkin 1968) As a consequence, the tissue blood exchange of tracer would be impaired which is in agreement with the present results These considerations suggest that the difference in the TFI values is partly due to the different abilities of the two types of vasodilatation to adjust the effective capillary surface area for optimal diffusion

A further explanation would be that the part of the disappearance curve during which the present measurements were performed, mainly reflects wash out of tracer localized in white muscle fibres (Tonnesen and Sejrsen 1970) with low oxidative metabolic activity (Romanul 1965) It has been shown in other species that the gastrocnemius muscle contains fibres with different oxidative metabolic activities, the number of capillaries surrounding each fiber being proportional to this activity (Romanul 1965) It may be assumed that the disappearance rate during the initial part of the disappearance run reflects events in both well perfused (red) and less well perfused (white) parts of the tagged muscle tissue (Tonnesen and Sejrsen 1970) However, high resting blood flow around the red fibres (Folkow and Halicka 1968, Hilton Jeffries and Vrbova 1970) would with time deplete tracer in well perfused areas (Reis, Wooten and Hollenberg 1967) which would result in tracer concentration gradients between white and red fibres In such situations the k value would predominantly reflect events around white fibres while the blood flow measurement would reflect events in the whole vascular bed Observations on mixed (gastrocnemius) and red (soleus) muscles indicate that metabolic vasodilatation increases blood flow about 300—400 % in mixed muscle while the increase in red muscle is considerably smaller 50—150 % (Folkow and Halicka 1968 Hilton *et al* 1970) Furthermore it was shown by the same authors that i a infusion of acetylcholine induced about the same relative increase in blood flow in the two types of muscle This mechanism would induce effects on TFI values similar to those we observed These considerations are also relevant to the response of TFI to reduction of blood flow

Concerning the quantitative aspects of xenon clearance, it has been found that during resting conditions the disappearance rate in the monoexponential phase underestimated total blood flow (Tonnesen and Sejrsen 1970). Our data support this observation. In a representative experiment (Fig. 1) total blood flow during control periods was 4.5 ml/min, while blood flow calculated from xenon data was 1.5 ml/min (assuming a partition coefficient of 0.7). Furthermore, as also can be seen from Fig. 1, the resting k values were higher for iodide than for xenon. This was seen in the majority of double tracer experiments. Thus in five of eight experiments the resting k values for iodide were higher than those for xenon and in one experiment they were equal.

In these experiments both tracers had been added to the same injection solution. Therefore, injection trauma and other methodological errors should have affected the two disappearance rates to the same extent. Xenon is more lipophilic than iodide and a high lipid content in the muscle would cause low xenon k values. This seems to be an unlikely explanation since a high lipid content would not only affect the xenon k value during resting conditions but also during stimulation. A more probable explanation is that the xenon molecules leave the vascular bed where the diffusion gradient goes from blood to tissue (as may be the case on the venous side outside the depot) but remain within the area monitored by the detector. This is largely in agreement with the hypothesis of shunting by diffusion put forward by Tonnesen and Sejrsen (1970).

There are thus several different possible explanations for the observed dissociation of tracer disappearance rate and blood flow. With the present technique it is not possible to state which of these is the more important. Two conclusions can be drawn. It appears possible to differentiate between vascular reactions induced by different means by combining tracer disappearance and blood flow measurements. Furthermore, the determination of blood flow from changes in xenon disappearance rate alone may give misleading results, especially when pronounced adjustments in capillary function occur. The assumption that xenon clearance is entirely blood flow limited should be critically evaluated in each application (cf. Kety 1960).

This investigation was supported by grants from the Swedish Medical Research Council (B7[illegible]-24X-816-04, B70-24X-816-05), National Institutes of Health, Bethesda, Md., U.S.A. (Training grant 5 TI HE 5314-05), Swedish Association of Medical Research, Karolinska Institutet, Stockholm.

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Material and Methods

Labelling procedure

Two or three adult rats of a hooded strain weighing 150—200 g, were used in each experiment Two narrow holes were drilled through the cranium under ether anesthesia, above the hemispheres and about 2—3 mm on each side of the median suture Labelled phosphate and inhibitor were injected through these holes Each animal was given 200 μCi of an isotonic solution of sodium ^{32}P-orthophosphate in phosphate buffer followed a few min later by an injection of 50 μl cordycepin in H_2O (1 μg/μl) The animals were killed by decapitation under slight ether anesthesia after various periods of time and the brains immediately removed

Isolation of nuclei and preparation of RNA

Nuclear isolation and fractionation were performed as previously described (Løvtrup-Rein and McEwen 1966) except that no Triton X 100 was used in the homogenization medium The techniques for RNA extraction and purification as well as for DNA removal, were the same as those described and discussed in an earlier report (Løvtrup-Rein 1970)

RNA fractionation

Nuclear RNAs were separated by an electrophoretic method on composite agarose-acrylamide ... blished by Weinberg ... thylene bisacrylamide ... ctively, according to ... the gels (1) 15 % ... iter), (2) 10 % am- ... hydroxy)methylamino methane 0 2 M sodium acetate 0 02 M ethylene diamine tetraacetate adjusted to pH 7 4 with acetic acid (4) 5 % sodium dodecyl sulphate (SDS) in water The composite gels (2.3 %

... (6 mm

... o (v/v) ... a tank ... el for 3 hrs When the run was completed the gels were scanned at 254 nm within the quartz tubes in a Vitatron densitometer

Measurements of radioactivity

After scanning the gels were removed from the tubes frozen and cut in 1 mm slices Each slice ... 1 200 μl NCS solubilizer After 2 hrs ... was measured in a Packard Tricarb ... (0 4 % 2 5-diphenyloxazole 0 01 %

Chemicals

Sodium ^{32}P-orthophosphate in isotonic phosphate buffer was purchased from the Radio- ... from Sigma Chemical Co Acrylamide ... hylenediamine were supplied by Koch ... Houses NCS solubilizer was a product of ... ck Sharpe and Dohme

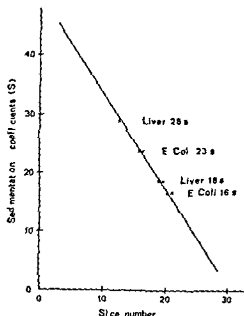


Fig 1 Correlation between electrophoretic mobilities and sedimentation coefficients of ribosomal RNA from rat liver and *E coli* Electrophoresis was performed on 2 3 % acrylamide—0 1 % agarose gels for 3 hrs at 4 mA per gel as described under Material and Methods Two independent experiments are represented by and x

Results

Determination of the S values of unknown RNA species

The correlation between electrophoretic mobilities and sedimentation coefficients as established by the use of rat liver and *E coli* ribosomal RNA as reference markers is shown in Fig 1 Two independent experiments performed on gels of different batches and ages are represented in order to show the reproducibility of the technique It is seen that a linear relationship prevails within the range of the standards as has previously been reported to be the case for pure agarose gels (Hadjiolov Venkov and Tsanev 1966) In the present context this linear correlation has been used for the estimation of sedimentation coefficients of unknown species even when these lie beyond the range of the markers This expedient probably involves a certain error which however is unlikely to be larger than the accuracy of the method

Inhibition of RNA synthesis by cordycepin in brain cell nuclei

Fig 2 3 and 4 show the effect of cordycepin on RNA synthesis in nuclei isolated from neurones astrocytes and other glial cells The radioactivity and UV absorbance profiles are represented With respect to the latter only the 28S and the 18S ribosomal components are clearly visible The radioactivity patterns present some features common for the nuclei of all three cell types and which are visible up to 60 min after the injection These are (1) the presence of small peaks of activity in the region between 45S and 28S which shift with time toward lower S values (2) the presence of two main peaks with sedimentation coefficients of 25S and 12S The first of these two species can often be seen as a small absorption maximum

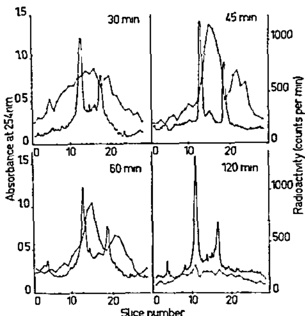


Fig 2 Effect of cordycepin on nuclear RNA from rat brain neurones at various times after addition of label and inhibitor ^{32}P phosphate and cordycepin were injected intracranially at doses of 200 μCi and 50 μg per animal respectively Nuclei were isolated and RNA extracted by hot phenol SDS as described earlier (Lovtrup-Rein 1970) RNA was fractionated by electrophoresis on 2 3 % acrylamide—0 1 % agarose gels for 3 hrs at 4 mA per gel Radioactivity was measured by liquid scintillation counting after solubilization of the gel slices Further details are given under Material and Methods Absorbance at 254 nm —, radioactivity —

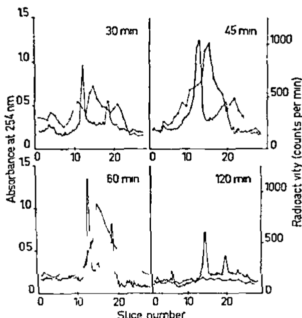


Fig 3 Effect of cordycepin on nuclear RNA from rat brain astrocytes Labelling and experimental conditions as described in Fig 2 Absorbance at 254 nm —, radioactivity —

Thirty min after the injection of label and inhibitor the 28S and 12S fractions are already clearly visible In the region above 28S however only small peaks of rapidly labelled RNA are observable

After 45 min action the activities in the two main components have increased further, while that of the 45S region has migrated towards the 35—32S area

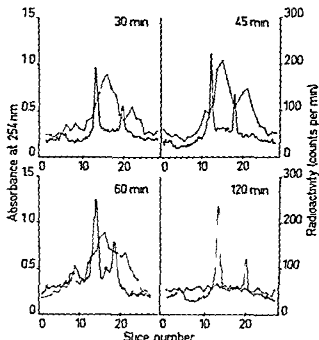


Fig 4 Effect of cordycepin on nuclear RNA from rat brain oligodendro- and microglial cells Labelling and experimental conditions as described in Fig 2 Absorbance at 254 nm, —, radioactivity ---

This shift of radioactivity from the high molecular weight fractions towards smaller components is still observable after 60 min At this time the activities of the 25S and 12S peaks are reduced and after 2 hrs completely flat profiles are obtained

It is worth noting that all the fractions described here appear and evolve in the same way in all three nuclear types However the oligodendro- and microglial nuclei are distinguished by an incorporation rate that is about 4 times lower than that found in the other nuclei

Discussion

In an earlier work on nuclear RNAs in brain cells Lovtrup Rein and Grahn 1970), we detected two groups of newly synthesized components In the first of these were high molecular weight fractions with sedimentation coefficients of 45S 38S 35S and/or 32S They underwent transformations which in other cell types were shown to lead to rRNA formation and we assumed them therefore to be ribosomal pre cursors Their synthesis and processing was delayed in non astrocytic glial nuclei as compared to the other nuclear types The second group comprised two species sedi menting at 25S and 12S characterized by similar rates of synthesis and turnover in all three types of nuclei The absolute amounts of all RNA fractions synthesized by oligodendro- and microglial nuclei were significantly lower

The use of a specific inhibitor of rRNA synthesis would make it possible to check the assumption stated above Cordycepin has been reported to inhibit selectively nucleolar RNA synthesis in HeLa cells (Siev Weinberg and Penman 1969) and to

suppress strongly the labelling of cytoplasmic RNA in Ehrlich ascites tumor cells (Frederiksen and Klenow 1964) The action of this drug on brain cells resulted in a great reduction of the components heavier than 28S Provided that the effect of this substance is the same on brain cells than on HeLa cells we may conclude that these RNA fractions are indeed ribosomal precursors Thus the contention that rRNA is formed at a lower rate in non astrocytic glial nuclei is also verified

The fact that small amounts of RNA are still observable in this region of the gradient is not incompatible with a selective inhibition of preribosomal fractions since it has been shown that some of the heterogeneous newly synthesized nucleoplasmic RNA sediments between 45S and 28S (Darnell 1968, Penman Vesco and Penman 1968 Warner *et al* 1966) Such heterogeneous RNA with DNA like base composition has been found in rat brain nuclei (Vesco and Giuditta 1967) It may be assumed that this type of RNA is not or only slightly affected by the inhibitor The possibility that the observed incorporation is the result of incomplete inhibition of preribosomal RNA synthesis can be ruled out since we cannot note in the present experiments the delay of formation of these components characteristic for non astrocytic glial nuclei

Comparison with our earlier results suggests that the 25S and the 12S species escape completely the action of the inhibitor This fact may indeed indicate that these two components are not of ribosomal type, although we still can not say whether they are messengers or not The radioactivity appears and decays simultaneously in both fractions No migration of label from the heavier to the lighter species could be noted We can therefore conclude that they exist as separate entities with no precursor product relationship Their rate of turnover is high since their activity reaches its maximum already after 45 min incorporation and has completely disappeared after 2 hrs

Although no difference could be observed between the three types of nuclei as to the rate of synthesis of heterogeneous nuclear RNAs and the 25S and the 12S fractions the absolute amounts of RNA formed in the nuclei from oligodendro- and microglial cells are significantly lower than those in the other nuclear types

The results presented here support all the assumptions made in earlier studies on the incorporation of radioactive precursors into the newly synthesized RNAs of fractionated brain cell nuclei Of particular interest were the two species which are shown here to be non ribosomal RNAs Rapidly labelled components with similar S values were detected by Tencheva and Hadjiolov (1969) in total brain RNA and DNA like RNA sedimenting between 6S and 30S has been observed by Stevenin Mandel and Jacob (1969) in rat brain cells Jacob *et al* (1966) reported the presence of a 25S component with messenger properties in nerve cells and Samli and Roberts (1969) found in brain cell nuclei a fraction ⩽ 12S which stimulates the incorporation of amino acids into ribosomal proteins If the existence of these fractions in brain cell nuclei is now beyond doubt their nature certainly is not so Further studies and possibly even a new methodological approach are required before any function can be attributed to these fractions

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From the Department of Anatomy, University of Helsinki, Finland

Uptake of 5-Hydroxytryptamine by Mast Cell Granules in vitro

By

S-E Jansson

Received 12 October 1970

Abstract

Jansson, S-E *Uptake of 5-hydroxytryptamine by mast cell granules in vitro* Acta physiol scand 1971 *82* 35—46

FCCP and which was not accelerated by adenosine triphosphate and magnesium chloride Reserpine and guanethidine affected little the endogenous 5-HT content but effectively depressed the uptake of 5 HT by the granules Amitriptyline and prenylamine lowered the endogenous 5 HT content and in some experiments depressed the uptake to some extent Imipramine, chlorpromazine, cocaine and g strophantine had little or no effect on the endogenous content or uptake of 5 HT It is concluded that the membrane free granules take up 5-HT from the medium by a passive process dissimilar from the active membrane dependent uptake mechanism in intact mast cells

It has been found that reserpine inhibits the uptake of 5 hydroxytryptamine (5 HT) by mast cells in vitro (Jansson 1970 a) The site of action of reserpine remained, however, in a previous paper (Jansson 1970 b) uncertain To solve this and related questions it was decided to study the uptake of 5 HT by isolated mast cell granules

Mast cell granules are easy to isolate with a high degree of purity (Lagunoff *et al* 1964, Thon and Uvnas 1966) and the isolated granules have been used in the study of the binding of histamine in mast cells (see Uvnas *et al* 1970) It has also been shown by Bergendorff and Uvnas (1967) that mast cell granules take up not only histamine and 5 HT, but also several other amines The uptake of histamine has been suggested to be due to a physical process dependent on extra- and intragranular concentrations of histamine and cations (Thon and Uvnas 1966, Uvnas *et al* 1970) To my knowledge the effect of drugs such as reserpine, prenylamine and guanethidine on the uptake and storage mechanisms of 5 HT in mast cell granules has not been as far investigated in vitro

Material and Methods

Peritoneal cells from adult Sprague-Dawley rats were used The cells were washed out from the abdominal cavity with 10 ml of an ice chilled Krebs Ringer solution modified by supplementing it with calcium chloride a
rats was pooled for every
sediment was resuspended
of NaOH (about 0 02 m
sediment was resuspended in 15 ml of fresh sucrose

Mast cell granules were isolated according to Lagunoff *et al* (1964) and Thon and Uvnas (1966) The cell suspension which consisted of leucocytes and about 3 % mast cells was frozen and thawed at least 3 times After the last thawing the temperature of the cell debris suspension was adjusted to 23° ...rature
which produced three f ... aterial
sedimenting at $2700 \times g$ ... ictions
are in the following refe

The sediments were resuspended in sucrose to give a protein concentration of about 350—750 (mean ± S D) µg/ml In several pilot experiments a "total sediment' was used consisting of material sedimenting at $2700 \times g$ for 30 min after freezing and thawing of the peritoneal cell suspension The incubations were carried out in a volume of 1 ml in 8 ml siliconized glass culture tubes The granule suspension was divided in an appropriate number of tubes To the

$2700 \times g$ for 30 min The pellet was washed by resuspension in 3 ml of sucrose and again collected by centrifugation

The 5 HT content of the sediment was determined spectrophotofluorometrically as described by Weissbach (1961) and explained in a previous paper (Jansson 1970) As a standard 5 hydroxytryptamine creatinine sulfate (Fluka) was used The concentrations refer to the 5 HT base

In some experiments the endogenous content as well as the uptake of 5 HT was expressed per mg protein (determined according to Lowry 1951) As it was noticed from the 5 HT/protein ratios that the granule containing sediment was contaminated by an extraneous protein the 5 HT values were routinely calculated on a percentual basis The effect of drugs on the endogenous 5 HT content (µg per sample) was calculated by expressing the 5 HT content of drug incubations in per cent of the 5 HT content (set at 100 %) of control aliquots of the same granule batch simultaneously incubated without drug The uptake of 5 HT was calculated by expressing the reached 5 HT (µg per sample) in per cent of the control content (aliquots of the same granule batch incubated without 5 HT) When the effect of drugs on the uptake was studied the uptake with drugs present (difference between original and reached 5 HT content µg per sample) was expressed in per cent of the control uptake (aliquots of the same granule batch incubated simultaneously without drug) calculated in a similar way and set at 100 %

The significance of the differences between means was calculated according to DeJonge (1964)

Two experiments with reserpine were performed in vivo In the first experiment 4 rats (about 200 g) were injected with reserpin 100 mg/kg subcutaneously (Serpasil, Ciba AG) 24
each rat for
ial from the
he sediment
4 test tubes
each The second reserpine experiment was identical with the first

With prenylamine (20 mg/kg dissolved in 50 % alcohol) 3 expts were performed in a similar manner with 3 animals in each experiment In every experiment an equal number of

C as pellets in 2 5 % glutar
and postfixed in
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specimens were embedded in ... were
with uranyl acetate and/or l
used

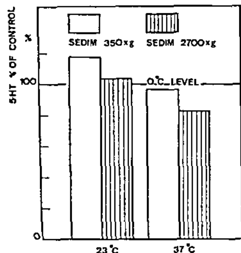


Fig 1

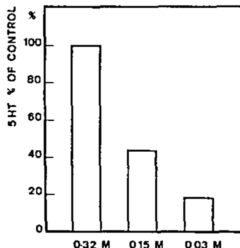


Fig 2

Fig 1 Effect of temperature on the endogenous 5 HT content of mast cell granules Samples incubated in isotonic sucrose pH 6 9 for one hour at 0° C 23° C and 37° C The 5-HT content of the samples was expressed in per cent of the samples incubated at 0° C (set at 100 %) Means of 4—6 expts

[illegible]

Means of 4 expts

Results

The differential centrifugation after freezing and thawing of the peritoneal cell suspension produced two particulate fractions 1) Sediment 350×g consisting of unbroken leucocytes partially degranulated mast cells and considerable amounts of extracellular mast cells granules, unbroken mast cells were rare, 2) Sediment 2700×g consisting of a practially pure mast cell granule fraction (Fig 4) contaminated only by an occasional nucleus a few eosinophilic granules and mitochondria After incubation with prenylamine chlorpromazine and guanethidine a precipitate was observed amongst the granules (Fig 5) The presence of soluble extraneous protein in this otherwise practically pure mast cell granule fraction was indicated by the amounts of 5 HT per mg protein (0 092 μg/mg) which was even smaller than that in the 350×g sediment (0 108 μg/mg) Lagunoff *et al* (1964) also reported the presence of such a protein

Of the 5 HT amounts recovered about 40 % was found in the supernatant while the two particulate sediments contained about equal amounts of 5 HT

The 5 HT content of the granules did not decrease upon incubation at room temperature for up to 6 hrs (Fig 10 control curve open circles) Neither was the 5 HT content affected to any notable extent upon incubation at 37° C or 23° C as compared to the 5 HT content upon incubation at 0° C (Fig 1) It was pointed

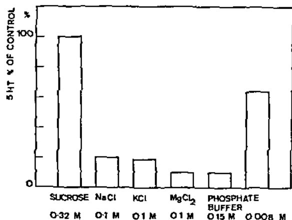


Fig 3 Effect of salts on the 5 HT content of mast cell granules Total sediment Granule material resuspended in salt solutions dissolved in isotonic sucrose Incubation one hour at 23° C 5-HT content in per cent of samples incubated in 0 3 M sucrose (set at 100 %) Means of 3 expts

by Thon and Uvnas (1966) that the suspension volume greatly affected the histamine content of the granules In the present study 7 times dilution was made without significant loss of 5 HT from the granules On the other hand, hypotonic solutions drastically decreased the 5 HT level (Fig 2)

As has also been found by Lagunoff *et al* (1964) and Thon and Uvnas (1966), the granules responded with pronounced loss of 5 HT to the addition of various salts to the incubation medium (Fig 3) Electron microscopically it was observed that the internal structure was loose and irregular upon incubation of the granules in 0 1 M NaCl (Fig 7), while the great majority of granules incubated in isotonic sucrose had a homogenous slightly particulate internal structure (Fig 4, 6) No limiting membranes around the granules were seen

Uptake of 5 HT by mast cell granules

On testing the uptake of 5 HT by mast cell granules as a function of the concentration of 5 HT in the medium using the same range previously tested for intact mast cells (Jansson 1970 a) it was found that only small amounts of 5 HT were taken up in an hour at low concentrations of 5 HT (0 22 and 0 44 μg/ml) This is at variance with results obtained with intact mast cells, in which these concentrations produced a maximal uptake The uptake by granules showed no saturation characteristics but the uptake increased with increasing concentrations of exogenous 5 HT for at least up to 17 6 μg/ml

In Fig 8 results with concentrations up to 4 4 μg/ml are given The experiments were performed at room temperature because as can be seen from Fig 9 the temperature of the incubation medium did not influence the uptake to any great extent at any concentration tested

At the concentration 4 4 μg/ml the uptake of 5 HT by the granule fraction increased with the incubation time approaching after 6 hours a suggested saturation level about 270 % of the original 5 HT content

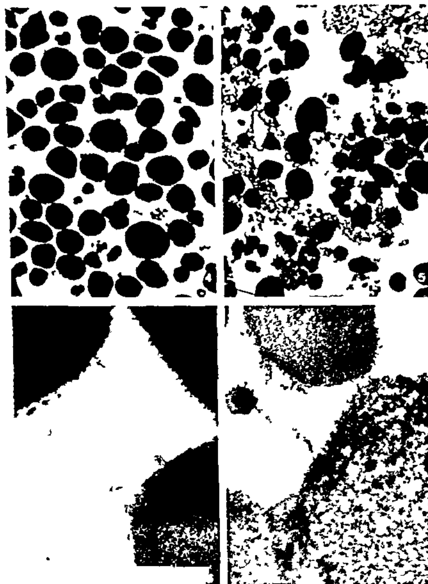


Fig 4 Electron micrograph of control granules in sediment 2700×g The fraction consists

right corner) has lost much of its electron density revealing a coarse internal structure At the periphery of the same granule only a fragment of a membrane is to be seen Magnification 100 000×

Fig 7 Electron micrograph of mast cell granules in sediment [illegible]00×g after exposure to NaCl at 10 [illegible] M for one hour at 23 C All granules are of decreased electron density as compared to the granules in Fig 6 One granule (lower right corner) shows an internal structure which apparently begins to crack up Magnification 100 000×

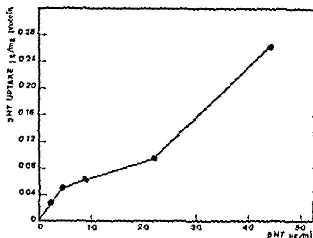


Fig 8 Uptake of 5 HT by mast cell granules as a function of the exogenous 5 HT concentration Sediment $2700 \times g$ Incubation one hour at 23° C Means of indicated number of expts

Metabolic inhibitors (sodium cyanide, dinitrophenole, FCCP[1] at 10^{-5} M) affected neither the endogenous content (92—104 % of the control content) nor the uptake of 5-HT to any notable extent (uptake with inhibitor at 10^{-5} M 100—118 % of the control uptake without inhibitor) Adenosine triphosphate (ATP) had no significant effect on the uptake (120 % of the control uptake, S E = ±20) while $MgCl_2$ at 2×10^{-3} M alone or together with ATP lowered the uptake of 5 HT (57 % of the control uptake)

Effect of drugs on the uptake of 5-HT

Of the amines tested only tyramine, and to a less extent tryptamine lowered the endogenous content of 5-HT (Table I) None of the amines was able to depress the uptake, indicating a preferential uptake of 5 HT over the other amines

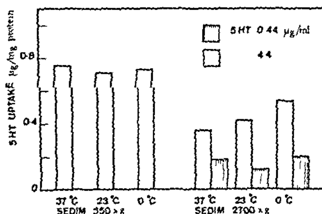


Fig 9 Uptake of 5 HT by mast cell granules as a function of temperature [illegible] for one hour [illegible]

[1] FCCP - [illegible] Wikström, [illegible] as a gift from P O [illegible]
Dept [illegible]
Heyll [illegible]

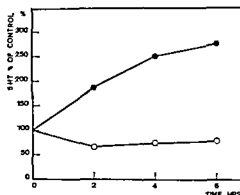


Fig 10 Uptake of 5 HT by mast cell granules as a function of time Total sediment 5 HT concentration in the medium 4 4 µg/ml Incubation at 23° C 5 HT content in per cent of the 5 HT content at zero time Means of 6 expts 5 HT, 4 4 µg/ml, ● Control 0

A few pilot experiments were performed with drugs used in earlier amine uptake and release experiments (Jansson and Penttila, 1969), using the total sediment obtained upon centrifugation at 2700×g for 30 min (see material and methods) The drugs were checked at 10⁴ M, a concentration at which no fine structure damage on the granules could be detected

Of the drugs tested chlorpromazine, amitriptyline and prenylamine lowered the endogenous content of 5 HT (29 %, 53 % and 52 %, respectively, of the control content without drug) Amitriptyline and prenylamine were also able slightly to depress the uptake of 5 HT by the granules (75 % of the uptake without drug) The uptake was depressed to the same extent by imipramine, while guanethidine had a clear inhibiting effect on the uptake (14 % of the uptake without drug) In these pilot experiments reserpine inhibited the spontaneous loss of 5 HT from the granules

TABLE I Effect of amines on the endogenous content and uptake of 5 HT by mast cell granules Pre incubation with 10 µg/ml of amine for 30 min at 23° C Further incubation with the same concentration of amine and 4 4 µg/ml of 5 HT for 1 hr at 23 C Total sediment The 5-HT content is expressed in nanograms per incubation sample Means of 2 expts

Drug	Endogenous 5-HT per cent of control content		Reached 5-HT content after incubation in 5 HT	Increase in 5 HT content = uptake per cent of control content		per cent of control uptake
	ng/sample	%	ng sample	ng sample	%	%
None (control)	48	100	64	16	33	100
Tryptamine	38	79	59	21	55	131
Tyramine	19	40	46	27	142	169
None (control)	85	100	122	37	44	100
Dopamine	93	109	129	36	39	97
α Methyl Noradrenaline	86	101	119	33	38	89

T II ET r

cubation sample Means of 2 experiments with prenylamine Means of 4 expts with the other drugs

Drug	Endogenous 5 HT		Reached 5-HT content after incubation in 5 HT	Increase in 5 HT content		uptake
	per cent of control content			per cent of endog content		per cent of control uptake
	ng/sample	%	ng/sample	ng/sample	%	%
None (control)	70	100	105	35	50	100
Reserpine	74	106	89	15	20	43
None (control)	90	100	144	54	60	100
Prenylamine	11	12	68	57	518	106
None (control)	26	100	110	84	323	100
Guanethidine	10	38	58	48	480	57
Imipramine	26	100	123	97	373	116

but because of an accident no information regarding the effect on the uptake was obtained Cocaine and g strophantine did not affect the endogenous content nor the uptake of 5 HT to any notable degree

The effects of reserpine prenylamine guanethidine and imipramine at 10^{-4} M was closer studied using the $350 \times g$ sediment containing mast cell fragments (Table II) as well as the $2700 \times g$ sediment mainly consisting of mast cell granules (Table III) The results showed that reserpine and guanethidine lowered the uptake as compared to the control uptake without drug in both sediments Reserpine exerted its action without affecting the endogenous content of 5 HT to any notable extent while guanethidine lowered the content to 40—70 % of the control content

Imipramine affected neither the endogenous content nor the uptake of 5 HT by the granules

Prenylamine drastically lowered the endogenous content of 5 HT and also depressed the uptake by sediment $2700 \times g$ as compared to the control uptake without drug but was without effect in this respect in sediment $350 \times g$

The effect of reserpine and prenylamine was therefore further compared in combined in vivo—in vitro experiments 24 hrs after a subcutaneous injection of reserpine (10 mg/kg) the endogenous content of 5 HT in intact mast cells was decreased to about half of the controls (Table IV) and the uptake of 5 HT by granules isolated from these cells was also clearly depressed (Table V) Prenylamine (20 mg/kg) behaved in quite an opposite way in slightly elevating the 5 HT content of intact mast cells and in being without notable effect on the uptake of 5 HT by granules isolated from mast cells exposed to the drug in vivo

TABLE III Effect of reserpine, prenylamine, guanethidine and imipramine on the endogenous content and uptake of 5-HT by mast cell granules Sediment II 2700 xg consisting mainly of membrane-free mast cell granules Experimental conditions as in Table II

Drug	Endogenous 5-HT per cent of control content		Reached 5-HT content after incubation in 5-HT	Increase in 5-HT content = uptake: per cent of endog content		Increase in 5-HT content = uptake: per cent of control uptake
	ng/sample	%	ng/sample	ng/sample	%	%
None (control)	90	100	179	89	99	100
Reserpine	88	98	137	49	56	55
None (control)	45	100	125	80	178	100
Prenylamine	16	36	56	39	244	49
None (control)	62	100	121	59	95	100
Guanethidine	45	73	80	35	78	59
Imipramine	47	76	113	66	140	112

TABLE IV Effect of reserpine (10 mg/kg) and prenylamine (20 mg/kg) on the endogenous content of 5 HT in rat peritoneal mast cells The drugs were injected subcutaneously 24 hrs before killing the animals Means and standard errors of 8—9 rats

Drug	Number of rats	5 HT, $\mu g/10^6$ Mast cells: Control	5 HT, $\mu g/10^6$ Mast cells: Drug	P
Reserpine	8—8	0 363±0 03	0 207±0 03	0 005
Prenylamine	9—9	0 562±0 11	0 685±0 10	

TABLE V Effect of reserpine (10 mg/kg) and prenylamine (20 mg/kg) administered in vivo on the in vitro uptake of 5 HT by mast cell granules Incubation with 5 HT at 4 4 µg/ml for one hour at 23° C. The granules were isolated from the rats in Table 4 5-HT expressed in nanograms per incubation sample The endogenous content and the uptake are not expressed in per cent of controls because the samples originate from different batches of rats

Drug	Endogenous 5-HT	Reached 5-HT content after incubation in 5 HT	Increase in 5 HT content = uptake: per cent of endog content	
	ng/sample	ng/sample	ng/sample	%
		Sediment 350xg		
None (control)	157	231	174	111
Reserpine	65	85	20	31
None (control)	39	83	44	113
Prenylamine	57	158	101	177
		Sediment 2700 xg		
None (control)	21	34	13	62
Prenylamine	30	45	15	50
None (control)	18	49	31	172
Reserpine	19	32	13	68

Discussion

According to Thon and Uvnas (1966) incubation in sucrose deteriorates the granule storage mechanism for histamine These authorities therefore *recommend to disrupt the cells in* distilled water and to perform the incubation of the isolated granules in water

In my hands disruption of the previously frozen and thawed mast cells and subsequent isolation of granules in water was hampered by a pronounced loss of 5 HT Because of this sucrose was used throughout the isolation and incubation procedures This might explain why in the present experiments about 20 % of the total 5 HT was retained in the granules upon exposure to various cations According to Thon and Uvnas (1966) granules incubated in sucrose have a decreased sensitivity towards cations

More difficult to explain is the drastic loss of 5 HT upon incubation in hypotonic solutions not observed by Thon and Uvnas This might again be due to the previous exposure to sucrose in the present study since Thon and Uvnas (1966) reported a reduced ability of the granules to retain the histamine content in hypotonic solutions after incubation in sucrose However Lagunoff *et al* (1964) did not observe any histamine loss in water after isolation of the granules in sucrose This discrepancy cannot be explained by the present study

As judged from the present results the uptake of 5 HT by isolated mast cell granules showed the following characteristics The uptake was not temperature dependent and showed no tendency to saturation during one hour even at high concentrations of exogenous 5 HT The uptake was not accelerated by the addition of Mg^{++} ions and/or ATP and it was not depressed by metabolic inhibitors These results could be taken to suggest that the uptake of 5 HT by isolated mast cell granules would be due to a passive process This suggestion is supported by the observations of Bergendorff and Uvnas (1967) and Thon and Uvnas (1966

Assuming a passive uptake by granules the active uptake of 5 HT in intact mast cells (Jansson 1970 a) most probably depends on a membrane activity or pump

Isolated mast cell granules showed in the present study no limiting membrane as has also been pointed out by Lagunoff *et al* (1964) and Thon and Uvnas (1966) The possibility cannot be excluded therefore that in intact mast cells active uptake may *take place in the membranes surrounding the granules intracellularly* To my knowledge there is no study in which isolation of membrane surrounded mast cell granules have been published

Of the drugs tested imipramine cocaine and g strophantine are known to inhibit the uptake of amines by the adrenergic axons by interfering with the membrane pump (see Carlsson 1966 Dengler *et al* 1961 Carlsson and Waldeck 1966) It is therefore understandable that these drugs did not depress in the present study the uptake of 5 HT by the membrane free mast cell granules although they have been shown to inhibit the uptake by intact cells (Jansson 1970 a c) apparently by interfering with the active uptake mechanism at the cell membrane

As judged from the present results, the only observation indicating that the uptake of amines by isolated granules should not be due to a passive process is the inhibition of uptake by reserpine and guanethidine According to our present knowledge, the effect of both of these drugs on the nervous tissue is exerted by interference with the energy requiring uptake mechanisms (see Lundborg and Stitzel 1967, Carlsson *et al* 1963) Guanethidine is taken up by the membrane pump and further incorporated into the storage organelles by the ATP Mg⁺⁺-dependent uptake mechanism, and reserpine inhibits the uptake by interfering with the energy requiring amine uptake mechanism It would be convenient to suppose that reserpine and guanethidine are interfering with some sort of an energy requiring uptake mechanism in isolated membranefree mast cell granules, too No convincing evidence for such a mechanism has been obtained (see also Thon and Uvnas 1966) The effect of reserpine and guanethidine on the granular uptake of 5-HT cannot therefore be explained and the present results suggest that both drugs depress amine uptake by mechanisms so far unknown

As judged from the combined in vivo—in vitro experiments of the present study the site of action of reserpine is at the granular level In these experiments prenylamine behaved in an exactly opposite way than reserpine did, even if prenylamine was able to depress the granular uptake to some extent in some of the in vitro experiments This might indicate that these two drugs affect the mast cell 5-HT by different mechanisms The ' reserpine-like" action of prenylamine observed by several investigators on nervous tissue (see various authors in 'Biochemical Aspects on Prenylamine , Biochem Appl 1068 Suppl 1) does not therefore seem to be valid on mast cells

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From the Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden

Release of Histamine and Formation of Slow Reacting Substance in the Cat Paw Induced by Compound 48/80

By

KJELL STRANDBERG

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Abstract

STRANDBERG, K. *Release of histamine and formation of slow reacting substance in the cat paw induced by compound 48/80.* Acta physiol scand 1971. *82.* 47—58.

A method for perfusion of cat paws with constant flow and temperature is described. This technique has been used to study the relationship between the efflux of histamine and slow

by cat serum was demonstrated. The results suggest that the initial reaction(s) activated by compound 48/80 is common for the release of histamine and the formation of SRS in the cat paw but that these processes then proceed independently.

Principles eliciting slow, sustained contractions of certain smooth muscles have been shown to appear in various tissues in connection with the release of histamine induced by antigen (Kellaway and Trethewie 1940, Brocklehurst 1960) and compound 48/80 (Paton 1951, Chakravarty, Hogberg and Uvnas 1959). These smooth-muscle stimulating principles have been designated "slow reacting substances" SRS. A correlation between the amounts of histamine and SRS appearing in the effluents from cat paws perfused with compound 48/80 (Chakravarty *et al.* 1959) as well as in sensitized guinea-pig tissues exposed to antigen (Chakravarty and Uvnas 1960, Boréus and Chakravarty 1960) has been demonstrated. The concomitant appearance of histamine and SRS indicated that both agents could be products of the activation of the same or closely linked enzymatic precesses in the mast cells. This assumption gained support when, in addition to histamine, small amounts of SRS were

detected after the incubation of an almost pure suspension of rat peritoneal mast cells with compound 48/80 (Uvnas and Thon 1959, Änggård *et al* 1963)

Analysis of the biological and chemical properties of SRS would be facilitated by access to large amounts of SRS The yields of SRS obtained by perfusion of cat paws with compound 48/80 are high compared to those obtained by the other experimental procedures cited (Änggård *et al* 1963) The present investigation was undertaken to determine optimal conditions for the formation of SRS in the cat paw perfused with compound 48/80 The relationship between the release of histamine and the formation of SRS has at the same time been further studied The experiments demanded a new experimental procedure which is reported

Experimental procedures

Cat pa t perfusion

The method used was a modification of the technique described by Hogberg Thufvessor and Uvnas (1956) Cats of either sex (1 5—5 0 kg) were killed by injecting 1 g of pentobarbital intraperitoneally Immediately after death the paws were cut off above the ankle joint and the main artery of each paw was cannulated with thin polyethylene tubing (Clay Adams PE 50 Th ... from blood with a salt solution (NaCl 154 mM ... 10 per cent (v/v) Sorensen phosphate buffer ... ereafter referred to as salt solut on, at room ... perfusion chambers (Fig 1) which were maintained at the desired temperature by water circulated by a pump from a reservoir connected to a thermoregulator Unless otherwise stated the paws were perfused simultaneously by means of constant rate (1 ml/min) infusion pumps (Mek Lab Konstruktioner Vastra Frolunda Sweden) The time required for preparation of the paws prior to perfusion was 30—60 min The effluents were collected during 20 min periods in calibrated glass tubes placed in ice water The samples were centrifuged (350 × *g* 4° C) to deposit any blood cells The supernatants e boiled for 5 min to avo d inactivation of SRS (see Results) and centrifuged From each supernatant two 0 5 ml samples were removed for histamine assay, the rest of the supernatant was used for assay of SRS

Wl en the influence of enzyme inhibitors on the yield of histamine and SRS was stud ed they were added in corresponding amounts to effluents from paws not perfused with inhibitor

Assay of histamine and SRS

Histamine was determined fluorometrically according to Shore Burkhalter and Cohn (1959) using an Aminco-Bowman spectrophotofluorometer and 10 mm cuvettes The amine values quoted are expressed in terms of the free base

SRS Tl e content of SRS in the effluents was determined by bioassay based on the l near log dose response relationship demonstrated for SRS on the isolated guinea pig ileum (Chakravarty 1959) Segments of terminal guinea pig ileum were suspended in aerated Tyrode solution containing atropine sulphate (1 μg/ml) and mepyramine maleate (1 μg/ml) The concentration of ... ference with ... present in the ... concentration ... the 4 ml org ... laboratory sta ... ml bath fluid ... as one SRS u ... contraction (... preparations stored at −20° C were used throughout the experiments No loss of activity was observed as checked by repeated threshold determinations

Statistical evaluation of data

Common methods of analysis of variance were used in the statistical calculation of results (Dixon and Massey 1957)

Materials

Compound 48/80 was generously supp[illegible]
Nialamide was a gift from Pfizer AB
calculated as the dried base was ob[illegible]
guanidine sulphate was purchased fro[illegible]
substances used were obtained from standard commercial sources

Results

Spontaneous release of histamine and formation of SRS

The present perfusion technique allowed a smooth control of the experimental conditions Constant temperature and flow was maintained by the use of temperature controlled chambers and constant rate infusion pumps Very little fluid was retained in the paws during the perfusion considering that a salt solution containing no albumin was used as perfusion medium (Fig 2, lower panel) The retention was usually most pronounced during the first 20 min period and it did not increase if compound 48/80 was infused

In conformation of earlier results(Högberg *et al* 1956, Chakravarty *et al* 1959) the effluents contained small amounts of histamine and rarely any SRS when the paws were perfused with the buffer alone (Fig 2, upper panel) The outflow of histamine and SRS were virtually constant during the entire perfusion period (100

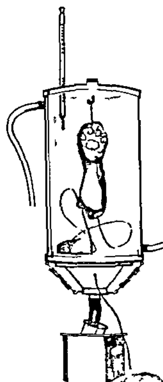


Fig 1 Perfusion of a cat paw mounted in a water jacketed temperature-controlled plastic chamber The effluent was allowed to ooze from the cut surface of the paw and to drain into a glass tube placed in ice water

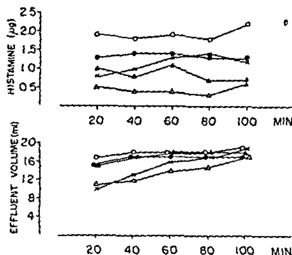


Fig 2 Efflux of histamine from cat paws perfused with salt solution alone at 27° C for 100 min Each curve represents one experiment In the upper panel, the symbols represent amounts of histamine in the effluents collected during 20-min periods In the lower panel, the volumes of the effluents are given No SRS was detected in any of these experiments

min) Hence in later experiments the paws were perfused with the salt solution for 20 min prior to the addition of compound 48/80 to allow temperature equilibration as well as to evalute the rate of spontaneous release of histamine and formation of SRS

Dose-response relationships

Diminished responses of the cat paws to repeated administrations of compound 48/80 were consistently noted In this respect the yields of histamine and SRS ... influenced similarly as shown in Fig 3 The figure also illustrates that the efflux of histamine precedes that of SRS

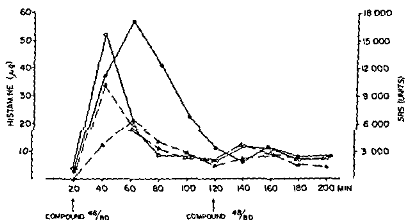


Fig 3 Influence of repeated administration of compound 48/80 on the efflux of histamine ... SRS from cat paws After a control period (20 min), the infusion of buffer (1 ml/min) ... jection of 5 µg (triangles) or 25 µg (circles) of com ... repeated 100 min later The injections are indicated ... of histamine (open symbols) and SRS (filled symbols) ... periods are given Two experiments are shown

TABLE I Efflux of histamine and SRS from cat paws perfused with compound 48 80 The values given represent the amounts of histamine and SRS in the effluents collected during 80 min with compound 48/80 (1 μg/ml) after perfusion with salt solution alone for 20 min

Exp no	Paw								Mean ± SEM	
	Left fore		Right fore		Left hind		Right hind			
	H	SRS	H	SRS	H	SRS	H	SRS	H	SRS
1	60 1	20,276	64 5	22,966	68 0	20 069	51 5	15 914	61 0 ± 3 5	19,806 ± 1 456
2	81 8	4,138	83 5	5,997	97 9	6,083	100 5	5 835	90 9 ± 4 8	5,513 ± 461
3	41 9	10,588	36 4	8 690	33 9	11,264	40 0	9,671	38 0 ± 1 7	10,053 ± 559
4	21 5	1,526	31 4	1,851	24 6	2 161	19 7	1,954	24 3 ± 2 5	1 873 ± 135
5	67 8	8,199	66 4	8 313	74 4	10,300	49 8	10,322	64 6 ± 5.2	9,258 ± 573
Mean ±	54 6	8 945	56 4	9,563	59 8	9 975	52 3	8,739		
± SEM	10 5	4,473	9 8	5,204	13 5	2,998	13 3	2,338		

H = μg histamine, SRS expressed in units

Intraarterial injections of high concentrations of compound 48/80 (50—100 μg) into cat paws often produced immediate but transient decreases in the effluent volumes Despite this disturbance, the yields of both histamine and SRS from these paws were usually about as high as from those paws where the corresponding amount of compound 48/80 had been infused during a period of 100 min To avoid interference with the experimental procedure the infusion technique has been used in the experiments below

Th output of histamine and SRS varied considerably from one cat to another whereas the paws of one and the same animal showed fairly similar responses to

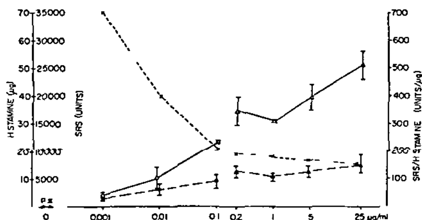


Fig 4 Concentration — response curves for the release of histamine and the formation of SRS in cat paws perfused with compound 48/80 at 27° C The amounts of histamine (open symbols) and SRS (filled symbols) in the effluents collected during 80 min perfusion with compound 48/80 after a control period of 20 min are given Each symbol represents the mean of 4 expts vertical bars denote standard errors The symbols to the extreme left represent the estimated spontaneous release of histamine and formation of SRS during 80 min, as calcula from the values of the control period SRS/histamine ratio x x

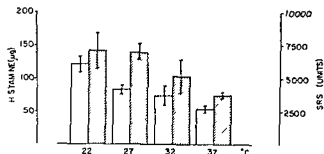


Fig 5 Influence of temperature on the efflux of histamine, □ and SRS, ▨, from cat paws perfused with compound 48/80 (1 µg/ml) for 80 min Each column represents the mean of 5 expts vertical bars denote standard errors

compound 48/80 (Table I) *No correlation between the yields of histamine and* SRS was noted even from paws of the same cat Fig 4 presents the dose-response *relations within two different dose ranges It appears that the cat paw is very* sensitive to compound 48/80 Thus the threshold dose was 0 001—0 01 µg/ml *for both histamine and SRS releasing effects The yield of histamine increased* relatively more with increasing concentrations of compound 48/80 than that of SRS *as illustrated by a decreasing SRS/histamine ratio*

The increase in the yield of histamine by increasing the concentration of releaser was *significant* ($p < 0\,01$), *as to SRS the increase was significant* ($p < 0\,05$) *only* in the lower concentration range

Influence of temperature

In five experiments paws of the same cat were perfused with compound 48/80 (1 µg/ml) for 80 min at 22° C, 27° C 32° C or 37° C The yield of both histamine and SRS was found to be influenced by the temperature (Fig 5) Most histamine appeared at 22° C The yield at 37° C was only 45 per cent of that at 22° C The yield of SRS was about the same at 22° C as at 27° C As for histamine the yields of SRS were lower at 32° C and 37° C Thus the yield of SRS at 22° C was about twice as high as that at 37° C The decline in yields by raising the temperature was significant (histamine, $p < 0\,001$, SRS, $p < 0\,01$) The time course for the efflux of histamine and that of SRS were not significantly influenced by the temperature (Fig 6 a, b)

The influence of temperature on the yields of histamine and SRS might be due either to a higher release of the substances at the lower temperatures and/or to an enhanced metabolic degradation at the higher temperatures In the case of histamine, the second alternative was investigated by studying the recovery of infused histamine and by attempting the inhibition of a presumptive histamine-degrading enzyme in the tissue When histamine (1 µg/ml) was infused at 22° C, 27° C, 32° C and 37° C, the loss of histamine ranged from about 30—40 per cent However, the recovery was not influenced by the temperature In another set of experiments, the release of histamine was induced by compound 48/80 (1 µg/ml) at 37° C and the influence of amino guanidine, an inhibitor of diamine oxidase (histaminase), and nialamide, a monoamino-oxidase inhibitor, on the yield of histamine was

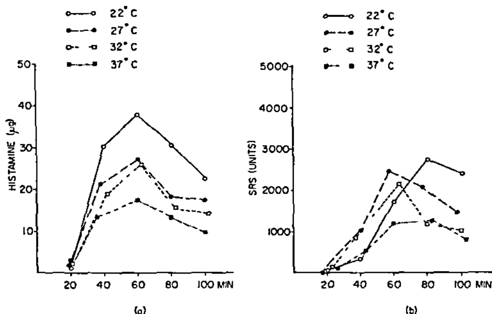


Fig 6 Influence of temperature on the time course for the efflux of histamine (a) and SRS (b) from cat paws perfused with compound 48/80 (1 µg/ml) The symbols represent amounts of histamine in the effluents collected during 20-min periods Each curve represents the mean of 5 expts

studied In contrast to compound 48/80 the enzyme inhibitors were present in the perfusion medium also during the first 20 min period of perfusion At 10^{-4} M no effects were observed

Inactivation of SRS by cat serum

It has been reported that guinea pig serum can inactivate SRS from cat paws (Änggård *et al* 1963) It was therefore considered of interest to study whether cat serum possesses a similar inactivating property Cat serum does in fact inactivate SRS Thus no SRS was found in the effluents from paws perfused with compound 48/80 (1 µg/ml) in 10 per (v/v) cat serum in salt solution although histamine release was not influenced The inactivating effect of cat serum was further studied by incubating SRS containing effluents with 2 per cent (v/v) cat serum for one hour at different temperatures A slight inactivation of SRS was detected in samples incubated at 4° C (Fig 7) More marked losses of spasmogenic activity were found at the higher temperatures However at the highest temperature 67° C no apperent inactivation was seen showing that the inactivating principle is thermo-labile

Influence of inhibitors

Amino groups reagents sulfhydryl group inhibitors and substances blocking cell respiration have repeatedly been reported to inhibit mast cell degranulation,

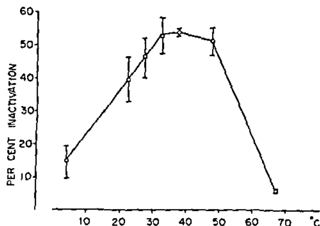


Fig 7 Influence of temperature on the inactivation of SRS by 2 per cent (v/v) cat serum in salt solution Incubation time one hour, controls were incubated with serum boiled for 5 min After incubation, all samples were boiled for 5 min The results are expressed as percentages of the activity of the controls Means and ranges of 2 expts

histamine release induced by compound 48/80 in rat tissues (see Uvnas 1964) In the present experiments the effect of one agent from each of these groups on the release of histamine as well as the formation of SRS in the cat paw was studied Ninhydrin, *N*-ethylmaleimide and potassium cyanide, all caused inhibition of the release of histamine and the formation of SRS induced by compound 48/80 (Table II) It was also observed that ninhydrin and *N* ethylmaleimide had a histamine releasing effect *per se* However, no SRS was detected in the effluents from paws perfused with these agents alone

Perfusion with histamine and heparin

Histamine releases glycerol and free fatty acids in canine subcutaneous adipose tissue (Fredholm, Meng and Rosell 1968) and heparin produces an increase in the lipoprotein lipase activity of the effluents from perfused rat hearts (Ho, Atkin and Meng 1966) Accordingly a study was made to determine whether the efflux of SRS from cat paws perfused with compound 48/80 could be secondary to a release of histamine or heparin However, when paws were perfused with histamine (1—10 μg/ml) or heparin (5—500 μg/ml), neither agent induced an efflux of SRS

Discussion

Compound 48/80 has been shown to induce the release of histamine and the formation of a lipid-soluble smooth-muscle stimulating material, SRS, in the cat paw (Hogberg *et al* 1956, Chakravarty *et al* 1959, Änggård *et al* 1963) In these experiments the paws were perfused by hydrostatic pressure and the temperature was maintained at the desired level by heating lamps In the present paper a method for perfusion of cat paws with constant flow in temperature controlled chambers is described This modification of the original technique (Hogberg *et al* 1956) yields reproducible experimental conditions and simplifies the experimental handling The

with inhibitors are expressed as percentages of the outputs from control paws perfused without inhibitors Means and ranges of 3—4 expts

Inhibitor, 10^{-4} M	20 min period		80 min period	
	histamine (µg)	SRS (units)	histamine (µg)	SRS (units)
Ninhydrin	603 (130—920)	100	45 (22—68)	30 (0—57)
N ethylmaleimide	1 807 (200—4 900)	67 (0—100)	80 (36—105)	38 (32—48)
Potassium cyanide	138 (83—191)	100	40 (27—54)	9 (0—35)

technique has allowed this study in which some experimental conditions favouring high yields of SRS have been defined

It has been shown that cat tissues, such as the cat skin flap (Feldberg and Paton 1951, Paton 1951) and the cat paw (Hogberg *et al* 1956, Chakravarty *et al* 1959), are sensitive to the histamine releasing effect of compound 48/80 Chakravarty *et al* (1959) also demonstrated *that an i a injection of a dose as low as 1 µg* of the releaser was sufficient to induce the formation of SRS in the cat paw In the present study compound 48/80 already at a concentration of 0 001—0 01 µg/ml induced an efflux of both histamine and SRS The yield of histamine increased with increasing concentrations of compound 48/80 within the range used (0 001—25 µg/ml) On the other hand the yields of SRS were not significantly higher when the concentration of the releaser was increased from 0 2 µg/ml to 25 µg/ml Hence, cat paws have been perfused with compound 48/80 at a concentration of 1 µg/ml in later experiments concerned with production of SRS for purification and characterization (Strandberg and Uvnas 1971)

Higher yields of both histamine and SRS were obtained at 22° C than at 37° C In the cat paw most histamine is located in the skin (Strandberg unpublished) The temperature optimum for histamine release in minced cat skin induced by compound 48/80 has been shown to be 30° C to 40° C (Westerholm 1960) The present results seem to be at variance with those of Westerholm (1960) Whether this is due to a higher release at 22° C than at 37° C in the present experiments and/or an enhanced metabolic degradation at the higher temperature cannot however, be concluded from the data The differences in experimental conditions have also to be considered

SRS was inactivated by cat serum The temperature dependence of the inactivation process suggests that it is enzymatic in nature It does not seem to be species specific since guinea pig serum has been shown to inactivate cat paw SRS (Ånggård *et al* 1963) To reduce the losses of SRS by such inactivation it is advisable to perfuse the paws thoroughly with salt solution to remove as much blood as po[illegible] prior to the introduction of the histamine releasing agent Furthermore the perf

should be chilled while collecting and then heated to destroy the inactivating property of any serum which may be present

Chakravarty *et al* (1959) have reported that variations in the experimental conditions *i e* changes in the concentrations of compound 48/80 preheating of the tissue exposure of the tissue to sulfhydryl group blocking agents, allicin or iodoacetate a polymer of salicylic acid (pk 11) and a polysaccharide fraction from hip seeds *similarly influenced the yields of histamine and SRS from cat paws The relationship* between the release of histamine and the formation of SRS in the cat paw induced by compound 48/80 was further studied in the present investigation On repeated administration of compound 48/80 the histamine releasing effect declined in parallel with a diminition in the output of SRS Moreover the yields of histamine and SRS were similarly influenced by the enzyme inhibitors used Thus when the histamine releasing effect of compound 48/80 was inhibited by ninhydrin *N* ethylmaleimide and potassium cyanide the yields of SRS were also reduced In addition these results indicate that both the release of histamine and the formation of SRS in the cat paw induced by compound 48/80 are dependent on energy requiring enzymatic reactions

Ninhydrin and *N* ethylmaleimide not only antagonized the histamine releasing effect of compound 48/80 but they also released histamine *per se* Hence the estimate of their true inhibitory effects was hampered The histamine releasing effect of *N* ethylmaleimide has been observed earlier in studies concerned with anaphylactic histamine release in rabbit basophil leucocytes (Greaves and Mongar 1968) and in guinea pig basophil leucocytes (Greaves 1969) Interestingly enough SRS was not detected in the effluents from paws perfused with ninhydrin or *N* ethylmaleimide one although histamine was found This means either that the formation of SRS was not evoked during the histamine release processes induced by these agents or that some step in its formation was blocked It should be noted in this connection that in guinea pig lung tissue the phosphatidase A induced formation of lipid soluble smooth muscle stimulating principles with biological properties similar to SRS is blocked by ninhydrin and N ethylmaleimide whereas the release of histamine is not influenced (Fredholm and Strandberg 1969)

The appearance of histamine in the effluents consistently preceded the efflux of SRS which is in accordance with earlier results (Chakravarty *et al* 1959 Anggård *et al* 1963) Chakravarty *et al* (1959) *suggested that this delay of the appearance* of SRS reflects different diffusion rates and/or the time period needed to produce SRS The finding that SRS in contrast to histamine could not be extracted from tissues not exposed to compound 48/80 constituted indirect evidence for the latter hypothesis If the delayed efflux of SRS is due mainly to its formation in the tissue the release of histamine is probably not dependent on formed SRS The lack of correlation between the yields of histamine and SRS even from paws of the same cat (Table I), agrees with this view

SRS appearing in the effluents from cat paws perfused with compound 48/80 has the chemical properties of a lipid soluble acid (Chakravarty *et al* 1959) Compound

48/80 has been shown to release glycerol and free fatty acids in canine subcutaneous adipose tissue probably due to liberation of histamine, since exogenous histamine produced the same effects (Fredholm *et al* 1968) Another mast cell constituent, heparin is considered responsible for the increase in lipoprotein lipase activity of the effluents from rat hearts, when they are perfused with compound 48/80 (Ho *et al* 1966) Since the efflux of histamine preceded that of SRS experiments were performed to determine whether histamine or heparin could induce the formation of SRS However, the efflux of SRS from cat paws does not seem to be mediated by released histamine or heparin, because infusion of either agent failed to produce any SRS

In summary, a method for perfusion of cat paws with constant flow and temperature has been developed This technique has been used to define conditions favouring high yields of SRS from paws perfused with the histamine releasing agent compound 48/80 It has also been shown that the release of histamine and the formation of SRS similarly are dependent on energy-requiring enzymatic reactions and decline on repeated administration of compound 48/80 This can be interpreted as support for the view, advanced by Chakravarty *et al* (1959), that the formation of SRS in the cat paw may be linked to the processes involved in the release of histamine However, the differences in the time courses as well as the lack of correlation between the yields of histamine and SRS seem to indicate two independent processes Possibly, the initial reaction(s) activated by compound 48/80 is common for both processes and then they proceed independently

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From the Department of Anatomy and the Department of Forensic Medicine, University of Helsinki, Finland

Effect of Some Drugs on the Uptake of Monoamines and Their Precursors in the Mouse Paneth Cells

By

A AHONEN and A PENTTILÄ

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Abstract

AHONEN A and A PENTTILÄ *Effect of some drugs on the uptake of monoamines and their precursors in the mouse paneth cells* Acta physiol scand 1971 *82* 59—69

The accumulation of some biogenic substances in the Paneth cell cytoplasmic granules of the mouse jejunum ileum and colon and the effect of some drugs on this accumulation were studied using the histochemical formaldehyde induced fluorescence method The biogenic substances were injected i v and the specific catecholamine fluorescence appeared in the cyto- [illegible] hydroxytryptophan were not taken up by the Paneth cell granules even after nialamide pretreatment The apparent mechanism of the accumulation of biogenic substances by the Paneth cell secretory granules was discussed.

Mammalian tissues contain various cell types capable of taking up catecholamines (CA) or their precursors This amine accumulation occurs not only in the adrenergic nervous tissue but also in certain cell types outside the nervous tissue Pearse (1966, 1969) has suggested that the capacity to synthesize monoamines and polypeptides carrying them in cytoplasmic granules and to bind monoamines is a peculiar characteristic also of a wide spread cell system of the body which Pearse calls APUD (Amine Precursor Uptake and Decarboxylation) cells

The specifity sensitivity and the reproductability of the formaldehyde-induced fluorescence method for histochemical demonstration of various biogenic monomines and their precursors is well understood (for ref see Eranko 1967) This method has also opened up new possibilities of studying the uptake of various biogenic substances at cellular level by cells which normally do not contain monoamines or their precursors One example of this kind of cell are the Paneth cells In the

normal mouse the cytoplasm of the Peneth cells exhibits no formaldehyde induced fluorescence, and therefore these cells can contain biogenic monoamines only in negligible amounts if some monoamine derivative exists in these cells This non fluorescent cytoplasm of the Paneth cells has made it possible in earlier studies (Penttila and Ahonen 1969, Ahonen and Penttila 1969, 1970) and in the present study to follow up the specific accumulation and binding of l-DOPA, dopamine nor adrenaline adrenaline and α methyl DOPA by these cells The greenish fluorescence seen in the cytoplasm of the cells after amine or amine precursor loading which was borohydride reactive and which faded quite quickly during UV-illumination, was interpreted as due to the condensation product of dopamine, l DOPA nor adrenaline, respectively with formaldehyde (see Corrodi and Hillarp 1963 1964, Corrodi Hillarp and Jonsson 1964)

Earlier reports by the present writers (Penttila and Ahonen 1969, Ahonen and Penttila 1969 1970) have shown that administered l DOPA, dopamine, adrenaline and noradrenaline are accumulated in the cytoplasmic granules of Paneth cells *in vivo* and that some drugs have a specific effect on the accumulation process It thus seems possible that the binding of catecholamines and their precursors by the secretory granules of Paneth cells may not be an unspecific loading process In the present study the effects of various drugs on the uptake of several amines or pre cursors were examined for further information on the accumulation and the binding mechanisms of biogenic substances by Paneth cells

Material and Methods

The material consisted of 300 white adult mice descendants of the strain used in the Department The animals were of both sexes They received no food for 24 hrs before experiments but were allowed to drink tap water *ad libitum* The mice were killed by decapitation in ether anaesthesia and the intestinal specimens were taken immediately after killing from the rostral end of the duodenum the first third of the jejunum the lower end of the ileum and the upper part of the colon The specimens contained all layers of the intestinal wall The tissue specimens were dissected in pieces of about 2 mm in diameter and 8 mm in length and were frozen in isopentane precooled in liquid nitrogen The specimens were then freeze-dried in vacuo at −40° C in a glass vessel with a phosphorous pentoxide layer on the bottom for two days and then warmed to room temperature in vacuo for five hours After drying the pieces were kept in formaldehyde vapour derived from paraformaldehyde at 80° C for 1 hr The relative humidity of the vapouring vessel was equilibrated at 70 % After immersion in xylene the specimens were mounted in paraffin wax 10 μ sections were cut and the paraffin wax was removed with xylene before viewing the sections in fluorescence microscopy The principles and details of the procedure are given in a practical review by Eranko (1967)

The following chemicals were injected slowly into the tail vein of the mouse l tyrosin (Fluka) α methyltyrosin Fluka l DOPA (Fluka α methyl DOPA (Merck Sharp & Dohme) dopamine Fluka) l noradrenaline hydrochloride (Medica) α methylnoradrenaline hydrochloride (Hoechst) adrenaline chloride Medica 5 hydroxytryptamine creatinine sulphate (Fluka) and 5 hydroxytryptophane phosphate Fluka

The following drugs were administered i p nialamide Pfizer pargyline hydrochloride (Abbot Lab), imipramine chloride Geigy ±) amphetamine sulphate (Orion amitriptyline hydrochloride Lundbeck nortriptyline hydrochloride (Lundbeck) chlorpromazine hydrochloride (Medica promazine chloride Star) guanethidine sulphate (Ciba) cocaine (Orion) and reserpine (Ciba

The drugs and chemicals were dissolved in an aqueous 0 9 % (w/w) sodium chloride solution 5-Hydroxytryptophan phosphate and l DOPA were dissolved with a minimal amount of aqueous 0 1 N HCl solution and gentle warming

The type of experiment, drug dose, action time and number of animals studied can be seen in Tables I to IV

Wild s monocular fluorescence microscope fitted with a high pressure mercury lamp HBO 200 (Osram) was used The light was filtered through Schott BG 12 and BG 23 filters and a Schott K 2 *heat absorbing filter The stop filters in the tube were* Leitz Euphos and Schott OG 1

The bright greenish formaldehyde-induced fluorescence of the delicate nerve fibres of the intestinal wall due to catecholamines and the distinct strongly yellowish fluorescent enterochromaffin cells due to 5 HT were used as histochemical criteria when evaluating the management of the freeze-drying procedure All the specimens which did not fulfil these properties were rejected

Numerous intestinal specimens were collected from several experiments for one freeze-drying procedure One or two mice in each experiment were used as controls and these animals received only saline via the tail vein When the combined effect of the biogenic substance and the drug was studied controls which received only the biogenic substance and controls receiving only the drug were made and the specimens from these animals were treated identically to those of the experiments in the freeze-drying procedure The effect of the drug on the accumulation of the biogenic substance by Paneth cells was evaluated by using these three types of control

Two or more pieces of duodenum, jejunum, ileum and colon were taken from each animal Two to five sections of each specimen were studied and the mean fluorescence intensity of 20—30 Paneth cells seen in these sections was estimated The fluorescence intesity was estimated visually in 10 μ sections by the present writers without previous knowledge of the section studied

Results

The Paneth cells of saline treated control mice did not show formaldehyde-induced fluorescence due to intrinsic biogenic monoamines or their precursors In the present study special attention *was paid to the Paneth cells located at the bottom of the* crypts of Lieberkühn, where they are most numerous The Paneth cells were more numerous in the ileum and the jejunum, where the cytoplasm of these cells also contained more secretory granules than the Paneth cells of the duodenum or the colon

Table I summarizes the mean fluorescence intensity of the Paneth cell cytoplasm after administration of various amines and amine precursors The homogeneous greenish fluorescence of the Paneth cell cytoplasm was visible already 5 min after intravenous administration of dopamine or l DOPA 10 min after injection of these substances, the greenish fluorescent material was mainly located in the coarse cytoplasmic granules of the Paneth cells As can be seen from Table I the cytoplasmic fluorescence of Paneth cells was visiable 150 min but not 4 or 6 hrs after dopamine administration Traces of greenish fluorescence were seen in the cytoplasm of Paneth cells 10 and 30 min after α methyl DOPA administration. In addition to the weak fluorescence intensity of the cytoplasm the number of fluorescent secretory granules was lower after α methyl DOPA than *e g* after l DOPA or dopamin administration. This effect was still more marked after noradrenaline administration

No formaldehyde induced fluorescence was seen in the cytoplasm of the Paneth cells 10 or 30 min after tyrosine α methyltyrosine, α methylnoradrenaline, 5-hydroxytryptophane or *5 HT* administration

Table II III and IV show the effect of various drugs on the accu[illegible] on amines and their precursors in the cytoplasmic granules of the Par[illegible]

TABLE I Accumulation of biogenic substances in the cytoplasmic granules of the mouse Paneth cells

Substance	Time (min)	Dose (mg/kg)	Number of mice	Intensity of fluorescence
Dopamine	—	—	3	—
Dopamine	10	16	4	—
Dopamine	10	40	4	+
Dopamine	10	160	8	++
Dopamine	30	160	6	++
Dopamine	60	160	4	++
Dopamine	150	160	4	+
Dopamine	240	160	4	—
Dopamine	480	160	4	—
l DOPA	—	—	3	—
l DOPA	10	160	4	++
l DOPA	30	160	4	++
Noradrenaline	—	—	3	—
Noradrenaline	10	160	4	—
Noradrenaline	30	160	4	+
Adrenaline	—	—	3	—
Adrenaline	10	160	4	—
Adrenaline	30	160	4	—
Tyrosine	—	—	3	—
Tyrosine	10	160	4	—
Tyrosine	30	160	4	—
α Methyl tyrosine	—	—	3	—
α Methyl tyrosine	10	160	4	—
Methyl tyrosine	30	160	4	—
α-Methyl DOPA	—	—	3	—
-Methyl DOPA	10	160	4	+
α Methyl DOPA	30	160	4	+
α-Methyl noradrenaline	—	—	3	—
α Methyl noradrenaline	10	160	4	—
α Methyl noradrenaline	30	160	4	—
5 Hydroxytryptophane	—	—	3	—
5-Hydroxytryptophane	10	160	4	—
5-Hydroxytryptophane	30	160	4	—
5 Hydroxytryptamine	—	—	3	—
5 Hydroxytryptamine	10	160	4	—
5 Hydroxytryptamine	30	160	4	—

The time means the interval between the administration of biogenic substance and the killing of the animal The fluorescence intensity was estimated visually as follows ++++ strong, +++ moderate, ++ weak, + trace of fluorescence and — no fluorescence

Paneth cell granules of the mice treated only with these drugs, using the same doses but omitting the biogenic substance, showed no formaldehyde-induced fluorescence, and the saline-treated controls were also negative

The monoamine oxidase inhibitors, nialamide and pargyline (Table II and IV)

TABLE II Effect of drugs on the accumulation of dopamine in the cytoplasmic granules of the Paneth cells

Drug	Time (hr)	Dose (mg/kg)	Biogenic Substance	Time (min)	Dose (mg/kg)	Number of mice	Intensity of fluorescence
Pargyline	3	200	—	—	—	3	—
Pargyline	3	200	dopamine	10	160	4	++++
Pargyline	3	200	dopamine	30	160	4	++++
Cocaine	0.5	100	—	—	—	3	—
Cocaine	0.5	100	dopamine	10	160	6	—
Cocaine	1.0	100	dopamine	30	160	4	—
Reserpine	24	10	—	—	—	3	—
Reserpine	24	10	dopamine	10	160	4	++
Reserpine	24	10	dopamine	30	160	4	++
Imipramine	0.5	100	—	—	—	3	—
Imipramine	0.5	100	dopamine	10	160	4	—
Imipramine	0.5	100	dopamine	30	160	4	—
Chlopromazine	0.5	100	—	—	—	3	—
Chlorpromazine	0.5	100	dopamine	10	160	4	++
Chlorpromazine	0.5	100	dopamine	30	160	4	++
Promazine	0.5	100	—	—	—	3	—
Promazine	0.5	100	dopamine	10	160	4	++
Promazine	0.5	100	dopamine	30	160	4	++
Amphetamine	0.5	100	—	—	—	3	—
Amphetamine	0.5	100	dopamine	10	160	4	++
Amphetamine	0.5	100	dopamine	30	160	4	++
Amitriptylen	0.5	100	—	—	—	3	—
Amitriptylen	0.5	100	dopamine	10	160	4	++
Amitriptylen	0.5	100	dopamine	30	160	4	++
Nortriptylen	0.5	100	—	—	—	3	—
Nortriptylen	0.5	100	dopamine	10	160	4	++
Nortriptylen	0.5	100	dopamine	30	160	4	++
Guanethidine	0.5	100	—	—	—	3	—
Guanethidine	0.5	100	dopamine	10	160	4	++
Guanethidine	0.5	100	dopamine	30	160	6	++

The first time column means the interval between the drug and dopamine administration The second time column means the interval between the administration of dopamine and the killing of the animal The fluorescence intensity was estimated visually as follows ++++ strong +++ moderate, ++ weak, + trace of fluorescence and — no fluorescence

markedly intensified the greenish formaldehyde induced flourescence of the Paneth cell granules after dopamine l DOPA or noradrenaline injection. Even after adrenaline and α methylnoradrenaline administration the accumulation of fluorescent substances in the Paneth cell granules was seen in UV microscopy when the animals were treated with MAO inhibitors but no such effect was seen after tyrosine, α methyltyrosine 5 HT or 5 HTP injection

TABLE III Effect of drugs on the accumulation of the biogenic substance in the mouse Paneth cells after nialamide pre treatment (500 mg/kg ip, 4—8 hrs before killing)

Drug	Time (min)	Dose (mg/kg)	Biogenic Substance	Time (min)	Dose (mg/kg)	Number of mice	Intensity fluorescence
Imipramine	30	100	—	—	—	3	—
Imipramine	30	100	dopamine	30	160	6	+
Imipramine	30	100	l DOPA	30	160	6	+
Chlorpromazine	30	100	—	—	—	3	—
Chlorpromazine	30	100	dopamine	30	160	4	+++
Promazine	30	100	—	—	—	3	—
Promazine	30	100	dopamine	30	160	4	+++
Amphetamine	30	100	—	—	—	3	—
Amphetamine	30	100	dopamine	30	160	4	++
Amphetamine	30	100	l DOPA	30	160	4	++
Amitriptylen	30	100	—	—	—	3	—
Amitriptylen	30	100	dopamine	30	160	4	++
Nortriptylen	30	100	—	—	—	3	—
Nortriptylen	30	100	dopamine	30	160	4	++
Cocaine	30	100	—	—	—	3	—
Cocaine	30	100	dopamine	30	160	4	+
Cocaine	30	100	l DOPA	30	160	4	+

The first time column means the interval between the drug and the biogenic substance administration The second time column means the interval between the biogenic substance administration and the killing of the animal The fluorescence was estimated as follows ++++ strong +++ moderate, ++ weak + trace of and — no fluorescence

Imipramine and cocaine markedly inhibited the dopamine and l DOPA uptake by Paneth cells whereas reserpine chlorpromazine promazine amphetamine, amitriptyline nortriptyline and guanethidine had no effect (Table II)

Table III shows the effect of some drugs on the catecholamine fluorescence of Paneth cells after nialamide pretreatment of the mice Imipramine and cocaine markedly inhibited the uptake of catecholamine precursors by Paneth cells normally *visible after nialamide pretreament Amphetamine amitriptyline and nortriptyline* inhibited similar uptake slightly after nialamide pretreatment although they did not prevent amine uptake without the MAO inhibitor

Reserpine chlorpromazine and promazine did not prevent amine uptake after nialamide treatment

Discussion

The secretory granules of the Paneth cells were first described by Schwalbe and later by Paneth The Paneth cells have been described in the intestine of man the rat, the rabbit, the mouse, and the guinea pig but they are not found in the

TABLE IV Accumulation of some biogenic substances in the mouse Paneth cells after nialamide pretreatment (500 mg/kg ip, 4—8 hrs before killing)

Biogenic *substance*	Time *(min)*	Dose *(mg/kg)*	Number *of mice*	Intensity of *fluorescence*
—	—	—	3	—
Dopamine	10	160	6	++++
Dopamine	30	160	4	++++
l DOPA	10	160	4	+++
l DOPA	30	160	4	++++
Noradrenaline	10	160	4	+
Noradrenaline	30	160	4	++
Adrenaline	10	160	4	—
Adrenaline	30	160	4	+
a Methyl DOPA	10	160	4	++
a Methyl DOPA	30	160	4	+++
a Methyl noradrenaline	10	160	4	—
a Methyl noradrenaline	30	160	4	+
l Tyrosine	10	160	4	—
l Tyrosine	30	160	4	—
a Methyl tyrosine	10	160	4	—
a Methyl tyrosine	30	160	4	—
5 Hydroxytryptophane	10	160	4	—
5 Hydroxytryptophane	30	160	4	—
5 Hydroxytryptamine	10	160	4	—
5 Hydroxytryptamine	30	160	4	—

The time means the interval between the administration of biogenic substance and the killing of the animal The fluorescence intensity was estimated visually as follows ++++ strong, +++ moderate, ++ weak, + trace of fluorescence, and — no fluorescence

intestine of some mammals and birds (Taylor and Flaa 1964 Pitha 1968) There are about 200 million Paneth cells in the human intestine but their contribution to the intestinal digestion process and their physiological significance are still uncertain (Toner 1968) In addition the secretion regulation mechanisms of these cells are unknown. Therefore the earlier (Penttila and Ahonen 1969 Ahonen and Penttila 1969, 1970) and present observations that the Paneth cells specifically accumulate amine precursors and catecholamines are interesting One possibility is that these substances may be connected with the synthetic and/or secretion regulatory mechanisms of the Paneth cells as suggested for the APUD cells storing and containing monoamines (Pearse 1969

5 min after dopamin or l DOPA administration a bright greenish homogeneous *fluorescence appeared in the Paneth cells cytoplasm and 5 min later the strongly* fluorescent material was seen mainly in the apical cytoplasmic granules of the cells. This accumulation time schedule indicates rapid transport of these two biogenic

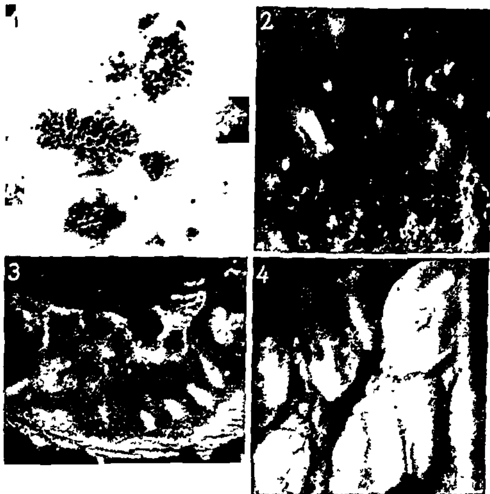


Fig 1 Formaldehyde-induced fluorescence in the mouse jejunum 30 min after dopamine administration (160 mg/kg) The mouse received a dose of 500 mg/kg nialamide 4 hrs before dopamine administration Distinct and strongly fluorescent secretory granules of the Paneth cells are to be seen in the apical region of the cytoplasm The figure shows the bottom area of a Lieberkuhn crypt, the section niveaus being in a transverse position to the longitudinal axis of the intestinal gland ×300

Fig 2 A similar experiment to that demonstrated in Fig 1 but the mouse received a dose of 100 mg/kg Imipramine 30 min before dopamine administration The inhibitory effect of Imipramine on the dopamine accumulation in Paneth cell granules can be seen ×150

Fig 3 Formaldehyde induced fluoresc
administration (160 mg/kg) Nialami
at the bottom of the crypts of Lieberk
a weak fluorescence The fluorescent
plasm of Paneth cells and coarse apic

Fig 4 Formaldehyde induced fluorescence in the mouse ileum 30 min after adrenaline administration (160 mg/kg) Nialamide pre treatment as in Fig 1 There are only traces of weak fluorescence in the apical cytoplasm of the Paneth cells and the cryptal lumen The section niveau of the crypt of Lieberkuhn is as in Fig 1 Three strongly fluorescent enterochromaffin cells are visible at the periphery of the crypts ×200

substances fram the capillary bed through the cell membrane into the cytoplasm and binding and storage of these substances by the Paneth cell granules The slow disappearance of dopamine from the Paneth cell granules, in contrast to the rapid disappearance from the elements of the surrounding mucous membrane suggests specificity of the storage and binding process by the Paneth cells After administration of noradrenalin or α methyl DOPA the granules were only weakly fluorescent and not all Paneth cells fluoresced The granule population of the Paneth cells seems to be heterogenous and possibly the binding capacity of the newly formed granules differs from those after maturation

In the present study the greenish fluorescence disappeared totally in 2 5 hrs after dopamine administration Sometimes greenish fluorescent cytoplasmic secretory granules were seen in the lumen of the intestinal crypts after l DOPA or dopamine administration Normally the Paneth cell granules are released into the intestinal lumen from the apical part of the cell in about 1—3 hrs (Toner 1968) It is therefore possible that the amines are transported with the granules into the intestinal lumen It is also possible that the accumulated amines participate in the synthetic or secretory process or both, and after liberation from the granular matrix the amines may be metabolized by monoamine oxidase whose activity is strong in the apical parts of the Paneth cells (Riecken and Pearse 1966)

A typical feature of the Paneth cells are their large apically located secretory granules derived from the Golgi vesicles (Hally 1958, Kurosumi 1961) The granules have a protein core containing substances with sulfhydryl groups, tyrosine trypto phan and arginine (Selzman and Liebelt 1961) Acid phosphatase and E-600 resistant esterase activity has been localized in the granuales, and the apical parts of the cells are rich in monoamine oxidase and zinc ions (Riecken and Pearse 1966) A specific feature of the granuale matrix is its extreme alkalinity (Taylor and Flia 1964, Merzel 1967)

The present results showed that cocaine and imipramine inhibited the uptake of dopamine and l DOPA by the Paneth cells The present and earlier observations on the Paneth cells (Penttila and Ahonen 1969) showed that monoamine oxidase in hibitor pretreatment intensified the cytoplasmic fluorescence and was able to prevent its disappearance The present results showed that this effect of nialamide on the Paneth cell fluorescence was only weak when cocaine or imipramine was injected before l DOPA or dopamine administration Cocaine and imipramine function at the cell membrane level (Isaac and Goth 1965, Glowinsky and Axelrod 1966) and the drugs are also able to inhibit the uptake of catecholamines by adrenergic nerves (Malmfors 1965) The present results possibly indicate a specific effect of these drugs on the Paneth cells and it seems obvious that the drugs function at the cell membrane level also in the Paneth cells Another suggestion of the specificity of the accumulation and binding mechanism of biogenic substances by the Paneth cell granules is that 5 hydroxytryptamine or 5 hydroxytryptophane were not taken up by these granules when studied in the same experimental conditions as catecholamines and their precursors 5 HT and catecholamines are equally metabolized

with monoamine oxidase It seems that 5 HT and 5 HTP are not bound by these granules and possibly are metabolized with the oxiding enzyme

In the central nervous system amphetamine is a weak blocker of the reserpine resistent catecholamine uptake (Fuxe and Ungerstedt 1968) Amitriptyline can inhibit the noradrenaline induced contractions of aortic strips (Scriabine 1969) In the present study amitriptyline and amphetamine had no effect on the catechol amine accumulation in the mouse Paneth cells, but after nialamide treatment they had a slight inhibitory effect.

The relationship between the amine formation and biosynthesis of polypeptides is a characteristic of the widespread cell system known as APUD (= Amine Pre cursor Uptake and Decarboxylation) cells proposed by Pearse (1969) These cells have been divided into two main groups The cells which store and presumably also produce 5 HT or catecholamines and the cells which normally contain neither 5 HT nor catecholamines but can be induced to store some arylalkylamines after loading the animal with the amine precursors There is no data on the presence or absence of the DOPA decarboxylase activity in the Paneth cells although it has been shown that there is a strong DOPA decarboxylase activity in the intestine and in the gastric mucosa (Håkansson, Owman and Sjoberg 1969) Therefore one of the problems ahead is to study the correlation of the DOPA decarboxylase activity to the Paneth cell cytoplasm and further to investigate the relationship of the Paneth cells to the APUD cell system

The following drugs were used

Reserpine (Serpasil®) and guanethidine (Ismelin®) Ciba
Nialamide (Niamid®) Pfizer
α metyl noradrenaline hydrochloride Hoechst Fennica
Pargyline hydrochloride Abbott Laboratories
α metyl DOPA (Aldomet®) Merck Sharp & Dohme
Imipramine chloride (Tofranil®) Geigy
Amitriptyline hydrochloride and nortriptyline hydrochloride Lundbeck
l Noradrenaline hydrochloride l adrenaline chloride and chlorpromazine hydrochloride Medica oy Finland
Cocaine Orion oy Finland
Promazine chloride Star oy Finland

For generous supplies of drugs we are indebted to above named companies

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From the Department of Pharmacology, University of Lund, Sweden

Action Potential Generation in Denervated Rat Skeletal Muscle

II. The Action of Tetrodotoxin

By

PAUL REDFERN[1] and STEPHEN THESLEFF

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Abstract

REDFERN, P. and S. THESLEFF. *Action potential generation in denervated rat skeletal muscle. II. The action of tetrodotoxin.* Acta physiol. scand. 1971. 82. 70—78.

Action potential generation was studied in the presence of tetrodotoxin (TTX) up to one week after denervation in individual muscle fibres of the extensor digitorum longus and diaphragm muscles of the rat. TTX 10^{-6} M completely blocked spike generation in innervated muscle and in muscle denervated for one day. On the second and subsequent days after denervation, action potentials resistant to TTX were recorded in the muscle fibres. In the extensor digitorum longus muscle denervated for 5 days action potentials recorded in the presence of TTX 10^{-6} M had a mean rate of rise 250 V/sec and they exceeded the zero membrane potential by an average of 28 mV. This TTX resistant action potential generation was impaired by reducing the external sodium concentration, and abolished in the absence of that cation. A correlation was observed between the presence of TTX resistant action potentials and the acetylcholine sensitivity of the muscle membrane.

It has been observed that after denervation the excitability of rat skeletal muscle is impaired. The main change was a reduction in the rate of rise of the action potential, which became manifest on the second day following denervation. It was not possible to relate the fall in rise rate to changes in the electrical time constant of the muscle fibre membrane and this suggested that structural changes had occurred in the membrane sites responsible for the inward sodium current of the action potential (Redfern and Thesleff 1971). To test such a possibility we compared the effects of tetrodotoxin (TTX) on spike generation in both innervated and denervated muscle. TTX is known to have a highly selective blocking action on the membrane channels which conduct sodium ions during the action potential (Kao 1966, Narahashi, Moore and Scott 1964).

[1] Present address: Department of Anaesthesia, P. O. Box 147, University of Liverpool, Liverpool, England.

Spike generation in denervated muscle was found to be partially resistant to the action of TTX It was also observed that this resistance was correlated to the development of acetylcholine sensitivity in the membrane Some of the results obtained have been published in a preliminary note (Redfern Lundh and Thesleff 1970)

Methods

The experiments were carried out on isolated extensor digitorum longus (E D L.) muscles of male Wistar rats with a body weight of 180 to 220 g The muscles were denervated unilaterally under ether anesthesia and at various intervals following this procedure the denervated muscle and its contralateral innervated control were removed and mounted together in an organ bath Experiments were also performed on E D L muscles in which surface fibers had been locally damaged by heat In frog muscle mechanical damage to muscle fibres is known to produce denervation like changes (Katz and Miledi 1964) Another series of experiments on the left hemidiaphragm denervated by intrathoracic section of the phrenic nerve was performed

The composition of the bathing fluid and the recording conditions were similar to those described previously (Redfern and Thesleff 1971) When the sodium concentrations was reduced osmolarity was maintained by the substitution of sucrose for sodium chloride When the ionic concentration was changed or drugs were added to the bathing fluid at least 30 minutes were allowed for equilibration before the experiment The acetylcholine sensitivity of denervated and of d m ged n l memb [illegible]

the results

To generate and record the action potential two microelectrodes were inserted into the same surface fibre about 50 μm apart one electrode was used for current passing the other to rec d [illegible]

advanced into the muscle fibre and used to trigger the action potential

Results

Effects of TTX on Action Potential

In agreement with the results of other workers we have found that action potentials of innervated rat skeletal muscle are greatly reduced in rate of rise and amplitude by TTX in a concentration of 10^{-8} M and cannot be elicited in TTX 10^{-6} M This is illustrated in Fig 1 A which shows the action potential in innervated E D L muscle in the absence of TTX Following the addition of TTX 10^{-6} M to the bathing fluid (Fig 1 C) cathodal currents to the fibre cause only depolarization without regenerative response We observed as previously described in frog muscle delayed rectification (Narahashi *et al* 1959) TTX had no effect on the resting membrane potential of the muscle

Following denervation the rate of rise of the action potential is reduced as described by Redfern and Thesleff 1971 and shown in Fig 1 B The addition of TTX to the bathing fluid in a concentration of 10^{-6} M fails to abolish the regenerative response although the rate of rise and the amplitude of the spike are reduced

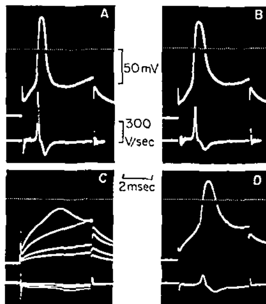


Fig 1 Intracellular recording of a typical action potential (upper trace) and its first derivative (lower trace) from an innervated (A) and a 4 day denervated (B) E.D L muscle Following the addition of TTX 10^{-6} M to the bathing fluid, action potentials could no longer be elicited in innervated muscle (C) whereas an action potential was still observed in the denervated muscle (D) The broken line in each trace indicates zero potential level of the cell Note the delayed rectification seen with the highest level of cathodal depolarization of the TTX blocked innervated muscle

1 D) Increasing the TTX concentration to 10^{-5} M, had no further effect on the spike, even when the drug was allowed to act on the muscle during several hours

As shown in Fig 2, the action potential in innervated and denervated muscle is equally affected by TTX in concentrations between 3×10^{-8} M and 3×10^{-7} M With TTX 3×10^{-6} M, action potentials cannot be elicited in innervated muscle, while that concentration has little further effect on the spike in denervated muscle TTX concentration of 3×10^{-8} M to 3×10^{-7} M reduced the rate of rise of the action potential in denervated muscle to the same extent as in innervated muscle (Fig 2)

The mean rate of rise of the action potential of 5 day denervated E D L muscle in TTX 10^{-6} M was 250 ± 51 v/sec, the action potential exceeded zero membrane potential by 28 ± 5.4 mV and the threshold for spike generation was 50 ± 4.0 mV

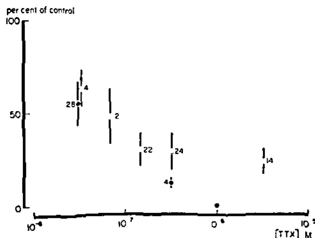


Fig 2 The effects of various concentrations of TTX on the rate of rise of action potentials of innervated (filled circles) and 3 days denervated (open circles) E D L muscles The means and standard deviations are expressed as a percentage of the values of the rate of rise in the same muscles in the absence of TTX, the number of control fibres being respectively 107 and 100 for innervated and denervated muscle The figures next to each mean value indicate the number of fibres examined

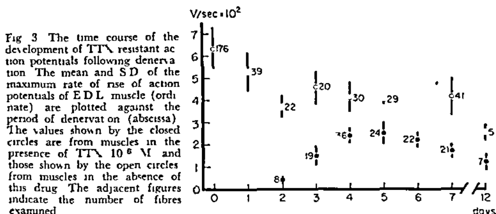


Fig 3 The time course of the development of TTX resistant action potentials following denervation The mean and S D of the maximum rate of rise of action potentials of E D L muscle (ordinate) are plotted against the period of denervation (abscissa) The values shown by the closed circles are from muscles in the presence of TTX 10^{-6} M and those shown by the open circles from muscles in the absence of this drug The adjacent figures indicate the number of fibres examined

TTX had no significant effect on the resting membrane potential of denervated muscle fibres which is reduced within 2 days of nerve section from over 80 mV to about 65 mV The TTX resistant action potentials were recorded in fibres locally polarized to −90 to −100 mV, and in the absence of this hyperpolarization cathodal stimulation failed to evoke action potentials in the presence of the drug In agreement with this finding we observed that TTX in a concentration of 10^{-6} M blocked the twitch generated by direct electric stimulation of the denervation muscle Even in the absence of TTX action potential generation is greatly reduced at the level of resting membrane potential found in denervated muscle (Redfern and Thesleff 1971), and this may explain why in the presence of the drug spikes were observed only in locally polarized fibres

Time Course of Development of TTX Resistant Action Potentials

To determine the onset of the appearance of TTX resistant action potentials E D L muscles were examined at various intervals following denervation As shown in Fig 3 regenerative responses resistant to TTX appeared on the 2nd day following denervation However at this time regenerative responses were only observed in some fibres (8 out of 19) and their rate of rise and amplitude were low On the third day of denervation action potentials were recorded in all fibres and by the 4th and 5th days their rate of rise reached a maximum of 200 to 300 V/sec. As the rate of rise of the action potential increased the threshold potential decreased and the amount by which the spike exceeded the zero membrane potential increased (Table I) There was little subsequent change in the spike The graph furthermore shows as previously described that the rate of rise of the action potential is reduced following denervation by about a third from about 600 V/sec to 400 V/sec

In experiments in which the muscle was allowed to become reinnervated following denervation by a nerve crush the addition of TTX 10^{-6} M completely prevented spike generation

TABLE I The table gives the mean values ± S D of resting membrane potential (R M P), rate of rise of the action potential, overshoot of the action potential and the threshold potential for spike generation in fibres of the E D L muscle at various intervals following denervation in the presence of TTX 10^{-6} M

Days of denervation	Number of fibres	R M P mV	Rate of rise v/sec	Overshoot mV	Threshold mV
0	26	81 ± 3 1	0	—	—
1	8	83 ± 3 6	0	—	—
2	[1]8/19	70 ± 5 4	40 ± 13	—	40 ± 2 7
3	19	61 ± 4 7	150 ± 44	15 ± 8 7	55 ± 5 4
4	36	59 ± 6 8	240 ± 33	26 ± 6 0	51 ± 3 1
5	24	69 ± 6 2	250 ± 51	28 ± 5 4	50 ± 4 0
6	22	68 ± 5 3	220 ± 34	25 ± 4 7	54 ± 3 5
7	21	64 ± 3 1	170 ± 33	25 ± 5 4	47 ± 2 8
12	7	64 ± 2 1	120 ± 38	18 ± 7 7	44 ± 2 4

[1] Action potentials were present in 8 fibres out of 19 penetrated Means and standard deviations are from the 8 action potentials

Correlation between TTX Resistant Action Potentials and Acetylcholine Sensitivity The question arose as to whether TTX resistant spikes coincide spatially with the presence of acetylcholine sensitivity in the muscle membrane Extrajunctional acetylcholine receptors develop following denervation (Axelsson and Thesleff 1959) and in innervated muscle after mechanical injury (Katz and Miledi 1964) A study was made, therefore of the relationship between TTX resistance and acetylcholine sensitivity in the denervated diaphragm muscle and in the innervated, locally injured, E D L muscle

Denervated diaphragm muscle As shown by Elmqvist and Thesleff (1960) the acetylcholine sensitive area of the end plate region of rat diaphragm muscle, starts to increase in size on the 2nd day following denervation Acetylcholine sensitivity gradually spreads towards the ends of the fibres and by one week after denervation, the entire cell membrane has a uniform and high sensitivity to acetylcholine

To study a possible correlation between electrical excitability and the spread of chemosensitivity, the diaphragm has the advantage that the individual fibres extend across the muscle from the central tendon to the rib insertion, with the end plate region in approximately the middle of the fibre The easily located end plate region makes it possible to determine whether the development of TTX resistant action potentials like that of acetylcholine sensitivity starts at the end plate and only subsequently spreads to the rest of the fibre

In innervated muscle and muscle denervated for 24 hours TTX blocked excitation in all parts of the muscle fibre As shown in Fig 4 and Table II however, TTX resistant action potentials could be recorded at the end plate region 2 days following denervation, while regenerative responses were absent from the rest of the fibre On the 3rd day, TTX resistant action potential generation improved in the end plate region and the first indications of a regenerative response occurred outside this region One week following denervation TTX resistant spikes were recorded from the entire length of the muscle fibres although the rate of rise was still somewhat higher

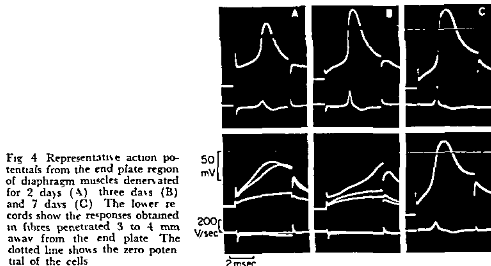


Fig 4 Representative action potentials from the end plate region of diaphragm muscles denervated for 2 days (A) three days (B) and 7 days (C) The lower records show the responses obtained in fibres penetrated 3 to 4 mm away from the end plate The dotted line shows the zero potential of the cells

potent als were recorded either from the former end plate region or from an area 3—4 mm d stant from the end plate

Days of denervat on	Areas penetrated	Number of fibres	R M P mV	Rate of rise V/sec	Overshoot mV	Threshold mV
WITHOUT TTX						
0	All	11	75±3 4	350±38	27±6 2	58±3 6
2	All	6	67±2 4	270±89	21±6 2	52±2 2
6—7	All	19	63±4 1	270±68	27±7 1	52±2 9
WITH TTX 10⁻⁶M						
0	All	5	70±3 7	0	—	—
2	End plate	9	58±3 3	100±47	11±11	43 5 1
	3 4 mm away	5	60±3 9	0	—	—
3	End plate	10	67±6 6	260 46	28 3 4	56 ? 8
	3-4 mm away	12	57±4 1	0	—	—
6—7	End plate	10	67±4 8	190±56	23±6 5	49 3 3
	3-4 mm away	15	61±4 1	110 29	11 8 1	48 3 9

in spikes recorded from the end plate region It thus appears that the development of TTX resistant action potentials following denervation has the same spatial and temporal distribution as that of acetylcholine sensitivity

As previously shown for the innervated end plate by Elmqvist and Feldman (1965) we observed that TTX had no significant effect on the sensitivity of the cholinergic receptors of denervated muscle to the action of acetylcholine It therefore appears that the sodium conductance increase induced by the acetylcholine [illegible] is insensitive to the blocking action of the drug

Locally injured E D L muscle Katz and Miledi (1964) have shown that local injury to innervated frog muscle fibres induces acetylcholine sensitivity in the membrane areas close to the site of injury This technique was therefore used to cause acetylcholine sensitivity in the innervated E D L muscle Surface fibres examined up to 7 days following thermal injury were found to be sensitive to acetylcholine at regions close to the injury, and also at a large area around the innervated end plate by three days after the injury In various parts of these fibres the presence of acetylcholine sensitivity was compared with their ability to produce a regenerative response in the presence of TTX 10^{-6} M Whenever a part of the membrane was found to have an acetylcholine sensitivity higher than about 1 mV depolarization per 10^{-8} Coulombs delivered by the pipette, *i e* about one thousandth of the sensitivity of the innervated end plate or of the chronically denervated muscle membrane a regenerative response was observed In other parts of the fibre TTX blocked the action potential

Effects of Some Neuromuscular Blocking Drugs on TTX Resistant Action Potentials

The correlation observed between acetylcholine sensitivity and TTX resistant action potentials suggested the possibility that acetylcholine receptors and action potential sites resided in the same membrane structures It was therefore of interest to see whether drugs with a specific action on acetylcholine receptors would also affect the TTX resistant action potential Gallamine and d tubocurarine which are competitive blockers of acetylcholine receptors in skeletal muscle failed to affect the action potential in concentrations up to 10 M In chronically denervated muscle decamethonium combines with cholinergic receptors depolarizing the membrane Subsequently however the decamethonium receptor combination leads to partial repolarization of the fibre by receptor desensitization (Thesleff 1955 delCastillo and Katz 1957) When decamethonium was used in concentrations of 10^{-3} M to 10 M action potential generation in the presence of TTX 10^{-6} M was greatly reduced or completely blocked Whether this action of decamethonium was secondary to changes in the ionic composition of the fibres resulting from the previous depolarization or the result of a direct action on spike generating membrane structures remains to be elucidated

Sodium Ion Concentration and Action Potentials in the Presence of TTX

It is well established that TTX blocks excitation when the action potential is initiated by the selective inflow of sodium ions but that the drug is ineffective on cells in which the spike is caused by the inflow of other ion species (Moore and Narahashi 1967) The question arose therefore as to whether the TTX resistant spikes of denervated muscle differed in such a way that the early transient increase in conductance was no longer specific for sodium The effect of changes in the external sodium and calcium concentrations on action current in denervated muscle was studied (Fig 5) Reduction of the external sodium concentration gradually reduced the rate of rise of the action potential and regenerative responses were almost absent when

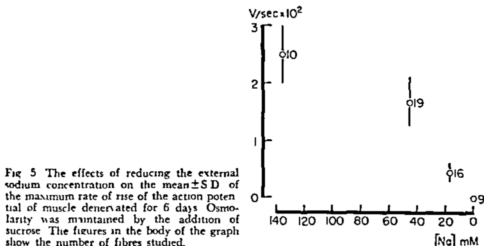


Fig 5 The effects of reducing the external sodium concentration on the mean ± S D of the maximum rate of rise of the action potential of muscle denervated for 6 days Osmolarity was maintained by the addition of sucrose The figures in the body of the graph show the number of fibres studied.

the sodium concentration was 17 mM (osmolarity was maintained by the addition of sucrose) Increasing the calcium concentration from 2 mM to 30 mM in the absence of sodium failed to restore a regenerative current

Discussion

The results of the present study have shown that, following denervation the action potential generating mechanism of skeletal muscle becomes partly resistant to the blocking action of TTX Quantitatively, TTX 10^{-6} M reduced the rate of rise of the action potential of denervated E D L muscle from about 400 v/sec to 250 v/sec, and the amount by which the spike exceeded zero membrane potential from about 40 to 25 mV

Spike generation in the presence of TTX was impaired in bath fluids containing a reduced concentration of sodium and spikes could not be evoked in the absence of external sodium Increasing the external calcium concentration did not restore regenerative responses and it appears therefore that even in the presence of TTX, sodium is still the main ion responsible for the rising phase of the action potential

Resistance to the blocking effect of TTX could follow from partial resistance of all spike generating membrane sites or from the presence of a proportion of sites totally resistant to the drug The observation that concentrations of TTX up to 3×10[illegible] M reduced the rate of rise of the action potential in denervated muscle to the same extent as in innervated muscle and the finding that increasing the concentration of TTX from 10^{-6} M to 10[illegible] M had no further effect on the spike in denervated muscle seems to support the possibility of some membrane sites being totally resistant to the drug

Since action potential generation is reduced following denervation it is unlikely that TTX resistant membrane sites for spike generation are formed in addition to

existing sites Presumably denervation induces TTX resistance in some of the pre existing sites perhaps with impairment of action potential generation at these sites

A correlation was observed between the development of acetylcholine receptors and the appearance of TTX resistant action potentials This applied to the diaphragm muscle as well as to innervated, locally injured, E D L muscle, showing that the receptor spread and the appearance of TTX resistant action potentials occurred with a similar time course and spatial spread The observation that TTX resistant spikes were present in innervated muscle fibres in which the acetylcholine sensitive area was enlarged following injury shows that denervation per se is not a prerequisite for this change

The nature of the change *responsible* for the development of TTX resistant action potential generation is unknown Further studies of the relationship between the presence of TTX resistant spikes and acetylcholine receptors will perhaps clarify this problem

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From the Department of Physiology, University of Göteborg, Sweden

Inotropic and Metabolic Effects of Dibutyryl Cyclic Adenosine 3', 5'-Monophosphate in the Perfused Rat Heart

By

Kurt Ahrén, Åke Hjalmarson and Olle Isaksson

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Abstract

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[illegible] were measured.

Dibutyryl cyclic AMP (5 × 10 [illegible] M and higher concentrations) evoked a positive inotropic effect as measured by an increase in peak systolic pressure when the heart rate was kept constant by electrical stimulation. The nucleotide also produced a marked and concentration dependent (10^{-5} to 10^{-3} M) glycogenolytic effect. Propranolol (10 [illegible] M) blocked the inotropic and glycogenolytic effects of adrenaline but did not reduce the effects of dibutyryl cyclic AMP. The effects of adrenaline were seen already 1 min after its addition to the perfusion medium, while the effects of dibutyryl cyclic AMP were not seen until 3—5 min after addition of the nucleotide. In time course studies, no dissociation was observed between mechanical and metabolic effects of the nucleotide.

The first evidence that adenosine 3',5'-monophosphate (cyclic AMP) might be involved in the positive inotropic effect of the catecholamines came from studies on phosphorylase activation in the perfused heart (for review see Haugard and Hess 1965). A good correlation was found between the activation of phosphorylase and the inotropic response, suggesting a common mediator for these effects. However, it was later shown in careful time course studies that the inotropic response preceded the time of onset of phosphorylase activation (Øye 1965, Robison *et al.* 1965). Furthermore, low concentrations of adrenaline could increase contractile force of the heart with no change in phosphorylase activity (Øye 1967). The finding that adrenaline stimulates adenyl cyclase activity in cardiac muscle homogenates indicates that cyclic AMP is involved in the inotropic response since the potency of catecholamines to stimulate adenyl cyclase activity is well correlated with their inotropic

effects *in vivo* (Murad *et al* 1962) Adrenaline was also found to increase the tissue level of cyclic AMP in the heart, and this increase preceded both the inotropic response and the activation of phosphorylase (Øye 1967, Robison *et al* 1965) The fact that theophylline, an inhibitor of the cyclic AMP degrading enzyme phosphodiesterase potentiates the inotropic effects of catecholamines also supports the hypothesis that an increased tissue level of cyclic AMP might trigger the inotropic response (Rall and West 1963)

Attempts to demonstrate an inotropic effect after addition of cyclic AMP to isolated atria of rabbits (Rall and West 1963) or guinea pigs (De Gubareff and Sleator 1965) have been unsuccessful Neither was any inotropic effect found after addition of cyclic AMP to the perfused rat heart (Robison *et al* 1965, Øye 1967) The inability to demonstrate an inotropic effect of exogenously administered cyclic AMP has been attributed to the low permeability of cardiac muscle to nucleotides Studies with labelled cyclic AMP have shown that this nucleotide does not accumulate in the rat heart to any significant extent (Robison *et al* 1965) Attempts to synthetize a derivate of cyclic AMP that penetrates the cell membranes more easily, have led to the compound N^6-2'-0 dibutyryl cyclic AMP This derivate of cyclic AMP has been effective in many tissues where cyclic AMP has failed to elicit a postulated effect However, even the dibutyryl derivate has been reported to be ineffective in evoking an inotropic effect in the perfused, working rat heart preparation (Robison *et al* 1965) On the other hand Kukovetz (1968) reported that addition of dibutyryl cyclic AMP to Langendorff preparations of hearts from rats rabbits and guinea pigs increased both their contractility and phosphorylase activity Recently dibutyryl cyclic AMP was found to increase the rate of tension development and the isometric tension of isolated cat papillary muscles and propranolol did not interfere with these effects (Skelton *et al* 1970) Injection of large doses of cyclic AMP to unanesthetized men increased heart rate and output (Levine and Vogel 1966), but these effects could have been reflexly induced

The purpose of the present investigation was to reinvestigate the effects of dibutyryl cyclic AMP in the perfused working [illegible] [illegible] o that of the effects of adrenaline The [illegible] of dibutyryl cyclic AMP and adrenaline were also studied in presence of the β-receptor blocking agent propranolol

Methods

Perfusion technique

Hearts were removed from fed female rats of the Sprague Dawley strain (80—90 g) that were maintained on a semisynthetic diet (Gustafsson 1959) and water *ad lib* [illegible] anesthetized with Nembutal 30 mg/kg b w [illegible] ice-cold saline The aorta and the left atrium [illegible] a working heart preparation as described by M[illegible] *et al* 1967) In contrast to the classical Lange[illegible] technique the left ventricle of the working heart preparation is pumping the perfusate against a hydrostatic pressure head of 70 cm H_2O and the heart is thus perf[illegible]ing an actual mechanical work *in vitro* Following a 5 min

retrograde washout period the hearts were moved to the working heart apparatus and were perfused in the forward direction

The perfusion was carried out using Krebs bicarbonate buffer (pH 7 4) continuously gassed with O_2 CO_2 (95 5 %) and containing glucose (2 5 mg/ml) The recirculating volume was 20 ml and the temperature was kept at 37° C The hearts were perfused for 1—60 min in presence of various concentrations of a monosodium salt of N⁶ 2′ 0 dibutyryl cyclic AMP (Boehringer & Soehne), adrenaline bitratrate (ACO) and dl propranolol (Inderal ICI) The left atrial filling pressure was 15 cm H_2O and the perfusion pressure 70 cm H_2O The aortic pressure and cardiac rate were recorded by a transducer attached to a manifold on the aortic cannula using a Sanborn recorder (model 16-1300 S) An air bubble of 1 5 ml was placed between the aortic Tygon tube and the transducer to provide some elasticity to the system Coronary flow was measured by collecting the fluid dropping from the heart chamber, and cardiac output was estimated by adding to the coronary flow the volume the heart was pumping to the top of the oxygenating chamber

The experiments were terminated by freezing the heart in a Wollenberger clamp cooled in liquid nitrogen, while the heart was still being perfused The frozen tissue was used for the analysis of glycogen, ATP and creatine P

Measurements of glucose uptake and lactic acid production

The glucose uptake of the heart was determined by measuring the initial and final glucose concentrations of the perfusion media with the glucose oxidase method (Saifer and Gerstenfeld 1958) The uptake is expressed as μmoles of glucose taken up per g of heart tissue (wet weight) per hour Lactic acid production by the heart was calculated from the amount of lactic acid found in the perfusion medium at the end of the perfusion period and was determined by an enzymatic method as described by Lundholm and co-workers (Lundholm *et al* 1963)

Measurements of glycogen, ATP and creatine P

For the determination of glycogen a piece of the frozen tissue was boiled in 30 % KOH for 15 min to extract the glycogen The glycogen was precipitated with Na_2SO_4 (8 %) and ethanol (100 %) and was thereafter enzymatically split into glucose by amylase, and the glucose was finally assayed by the glucose oxidase method Glycogen from rabbit liver was used as a standard For estimation of ATP and creatine P the frozen heart was homogenized and extracted with perchloric acid The extract was neutralized with K_2CO_3 and assayed by an enzymatic method involving hexokinase, glucose 6 phosphate dehydrogenase and creatine kinase (Lamprecht and Trautschold 1963)

Statistical procedure

Mean values are given ± standard error of the mean Comparison between different groups was performed according to Student s t test A p-value of 0 05 or less was considered significant in this study

Results

Effects of dibutyryl cyclic AMP on heart function

Hearts were perfused for 60 min with various concentrations of dibutyryl cyclic AMP At concentrations of 5×10^{-5} M or higher a concentration dependent increase in peak systolic pressure was obtained (Fig 1 and Table I In contrast to the rapid effects seen after administration of adrenaline there was a time lag of 3—5 min before the increase in peak systolic pressure was detectable After about 10 min the increase in peak systolic pressure had reached a maximal level and only a slight decrease from the maximum was seen during 60 min of perfusion Dibutyryl cyclic AMP did not accelerate the heart rate and in fact at the higher concentrations the heart rate was slightly decreased With the highest concentration of the nucleotide (10^{-3} M) the heart rate (beats/min was 235 ± 5 (8) compared to 263 ± 11 (8) for the controls ($p < 0.05$) The fact that any agent with a negative chronotropic effect

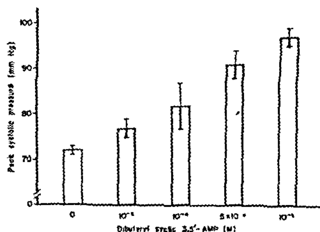


Fig 1 Effects of increasing concentrations of dibutyryl cyclic AMP on peak systolic pressure of perfused working rat heart The hearts were perfused for 60 min with Krebs bicarbonate buffer, containing glucose (2.5 mg/ml)

Each bar represents the mean of 6—9 observations and standard errors are indicated by vertical lines All dibutyryl cyclic AMP concentrations tested, except for 10^{-5} M, gave significant effects ($p<0.05$)

TABLE I Effects of dibutyryl cyclic AMP in absence and presence of propranolol on function, glycogen content and lactic acid production of perfused working rat heart

Group	Control		Dibutyryl cyclic AMP (5×10^{-4} M)	Propranolol ($10^{-[illegible]}$ M)		Dibutyryl cyclic AMP (5×10^{-4} M) + Propranolol ($10^{-[illegible]}$ M)
Heart rate beats/min	248 ± 17	N.S.	276 ± 13	233 ± 9	N.S.	230 ± 13
Peak systolic pressure mm Hg	72 ± 2	$p<0.025$	80 ± 2	78 ± 2	$p<0.05$	89 ± 3
Coronary flow ml/min	6.2 ± 0.4	$p<0.005$	8.0 ± 0.2	7.2 ± 0.2	N.S.	7.6 ± 0.2
Cardiac output ml/min	24 ± 0.5	$p<0.05$	31 ± 2	24 ± 1	$p<0.025$	23 ± 1
Glycogen content mg/g w.w.	1.82 ± 0.03	$p<0.02$	1.20 ± 0.19	1.94 ± 0.05	$p<0.001$	0.95 ± 0.13
Lactic acid production µmoles/g w.w.	34 ± 7	N.S.	34 ± 5	24 ± 5	N.S.	31 ± 9

Values are means ± S.E.M. There were 4 hearts in the propranolol group and 5 hearts in the other groups. Hearts were perfused for 60 min with Krebs bicarbonate buffer containing glucose (2.5 mg/ml). The values for the mechanical performance given in the table were recorded after a perfusion period of 30 min.

might indirectly cause an increase in peak systolic pressure made it difficult to evaluate an inotropic effect of dibutyryl cyclic AMP. However, when hearts were perfused with a lower concentration of dibutyryl cyclic AMP (5×10^{-5} M) the heart rate was not changed and increases in peak systolic pressure and cardiac output were recorded (Table I). In order to further evaluate whether the increase in peak systolic pressure produced by the higher concentration of dibutyryl cyclic AMP was due to its negative chronotropic effect hearts were electrically paced at two different fre-

TABLE II Effects of dibutyryl cyclic AMP on peak systolic pressure in perfused rat hearts beating at constant rates

Group	Heart No	Before electrical stimulation		During electrical stimulation	
		Heart rate beat/min	Peak systolic pressure (mm Hg)	Peak systolic pressure (mm Hg) at the heart rate of 250 min	300 min
Control	1	250	74	72	65
	2	250	69	72	66
	3	230	74	68	61
	4	210	90	74	66
Mean±S E M		235±10	77±5	72±1	65±1
Dibutyryl cyclic AMP (10^{-3} M)	1	220	92	94	89
	2	230 (S)	94 ($p<0.02$)	90 ($p<0.001$)	86 ($p<0.001$)
	3	180	94	87	76
	4	200	95	92	81
Mean±S E M		208±11	94±1	91±1	83±3

Hearts were perfused with Krebs bicarbonate buffer containing glucose (2 5 mg/ml) in absence and presence of dibutyryl cyclic AMP under a control period of 30 min The prestimulated value of peak systolic pressure was then registered The hearts were thereafter paced with an electronic stimulator (5 V at 1 msec) for 2—3 min at frequencies of 250 and 300 imp/min respectively Individual values for peak systolic pressure of 4 hearts in each group are presented in the table

quencies in absence and presence of the nucleotide (Table II) The peak systolic pressures of the hearts perfused with the nucleotide was considerably higher than those of the control hearts at the two different heart rates This shows that the increase in peak systolic pressure by dibutyryl cyclic AMP cannot be explained as secondary to its negative chronotropic effect

Effects of dibutyryl cyclic AMP on carbohydrate metabolism

Perfusion with dibutyryl cyclic AMP in concentrations from 10^{-5} to 10^{-3} M for 60 min resulted in a marked concentration dependent decrease in the glycogen content of hearts (Fig 2) The glycogen content decreased from 1 82±0 15 mg/g w w in

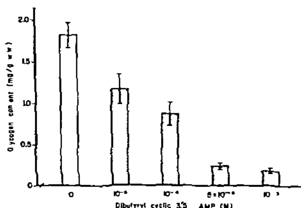


Fig 2 Effects of increasing concentrations of dibutyryl cyclic AMP on total glycogen content of isolated working rat heart perfused for 60 min with Krebs bicarbonate buffer containing glucose (2 5 mg/ml)

Each bar represents the mean of 7—8 observations and standard errors are indicated by vertical lines All dibutyryl cyclic AMP concentrations tested gave significant effects ($p<0.02$)

TABLE III Effects of adrenaline and dibutyryl cyclic AMP on heart rate, peak systolic pressure and

Min of perfusion[1]	No	Control			No	Adrenaline (7 5 × 10⁻⁷ M)	
		Heart rate beats/min	Peak systolic pressure mmHg	Coronary flow ml/min		Heart rate beats/min	Peak systolic pressure mmHg
10	4	245 ± 17	72 ± 3	6 4 ± 0 4	4	258 ± 16	68 ± 2
+1		245 ± 17	71 ± 3	5 6 ± 0 5		323 ± 18*	71 ± 2
10	6	270 ± 14	71 ± 2	7 1 ± 0 2	6	275 ± 9	67 ± 3
+2		275 ± 14	70 ± 3	7 2 ± 0 2		325 ± 16**	72 ± 2
10	4	243 ± 30	73 ± 5	6 1 ± 0 8	3	277 ± 12	76 ± 4
+5		266 ± 25	76 ± 5	5 7 ± 0 6		333 ± 3**	77 ± 4
10	7	254 ± 15	76 ± 2	5 9 ± 0 3	7	267 ± 9	78 ± 2
+10		264 ± 14	77 ± 2	6 3 ± 0 4		319 ± 12***	79 ± 3

the control hearts to 0 18 ± 0 03 in hearts perfused with dibutyryl cyclic AMP (10^{-3} M) A significant increase in lactic acid production was seen in the hearts perfused with higher concentrations of dibutyryl cyclic AMP The lactic acid production increased from 31 ± 3 μmoles/g w w in the control hearts to 54 ± 4 (8 hearts, $p < 0\ 001$) in hearts perfused with dibutyryl cyclic AMP (10^{-3} M)

Effects of dibutyryl cyclic AMP in absence and presence of propranolol

It can be seen from Table I that propranolol at a concentration of 10^{-5} M was completely ineffective in inhibiting or reducing the increase in peak systolic pressure and cardiac output produced by dibutyryl cyclic AMP Propranolol was further unable to reduce the decrease in glycogen content obtained in hearts perfused with dibutyryl cyclic AMP However the decrease in glycogen content produced by adrenaline ($7\ 5 \times 10^{-7}$ M) was significantly reduced by propranolol (10^{-5} M) The glycogen content was 0 95 ± 0 13 mg/g w w after perfusion for 60 min with adrenaline and 1 40 ± 0 10 mg/g w w after perfusion for 60 min with adrenaline and propranolol The chronotropic and inotropic effects of adrenaline were also blocked by this concentration of propranolol

Effects of adrenaline and dibutyryl cyclic AMP in short time experiments

In order to study the time course of the effects of dibutyryl cyclic AMP and adrenaline short time experiments were performed All hearts were perfused for 10 min before addition of adrenaline or dibutyryl cyclic AMP in order to stabilize heart rate peak systolic pressure and coronary flow It can be seen from Table III that adrenaline significantly accelerated the heart rate already after 1 min No significant increase in peak systolic pressure with adrenaline was seen in the present study, which might be explained by the increased heart rate and shortened time for

coronary flow of the perfused working rat heart

	No	Dibutyryl cyclic AMP (10^{-3} M)		
Coronary flow ml/min		Heart rate beats/min	Peak systolic pressure mmHg	Coronary flow ml/min
5.6 ± 0.5 5.5 ± 0.5	4	285 ± 19 285 ± 19	70 ± 5 67 ± 4	6.2 ± 0.3 6.1 ± 0.2
6.0 ± 0.5 6.5 ± 0.5	6	267 ± 15 252 ± 17	73 ± 3 73 ± 3	7.2 ± 0.2 6.3 ± 0.5
6.3 ± 0.3 7.2 ± 0.1*	3	300 ± 17 240 ± 6*	72 ± 3 99 ± 4**	5.7 ± 0.6 6.5 ± 0.4
6.3 ± 0.2 6.7 ± 0.2*	6	260 ± 14 230 ± 7	79 ± 2 105 ± 6***	6.0 ± 0.3 6.9 ± 0.2*

mg/ml) Thereafter, 0.5 ml of Krebs bicarbonate buffer alone (control), or the same amount of 10^{-1} M), or buffer containing dibutyryl cyclic AMP (to give a final concentration of 10^{-3} M) was Values are mean ± S E M P values vs the control value of the hearts in the same group before the

TABLE IV Effects of adrenaline and dibutyryl cyclic AMP on glycogen content, lactic acid production, ATP and creatine P content of the perfused working rat heart

Min of perfusion	No	Group	Glycogen content mg/g w w	Lactic acid production μmoles g w w × hr	ATP μmoles/g w w	Creatine P μmoles g w w
10+1	4	Control	2.12 ± 0.17	−55 ± 12	2.43 ± 0.17	2.90 ± 0.36
	4	Adrenaline (7.5 × 10^{-7} M)	1.32 ± 0.25*	752 ± 162**	1.78 ± 0.17*	1.53 ± 0.12*
	4	Dibutyryl cyclic AMP (10^{-3} M)	1.83 ± 0.12	−48 ± 20	2.47 ± 0.28	2.91 ± 0.34
10+2	6	Control	2.01 ± 0.14	−26 ± 13	3.05 ± 0.12	
	6	Adrenaline (7.5 × 10^{-7} M)	1.21 ± 0.16**	422 ± 40***	2.08 ± 0.26**	
	6	Dibutyryl cyclic AMP (10^{-3} M)	2.00 ± 0.11	48 ± 36	3.19 ± 0.22	
10+5	4	Control	1.99 ± 0.18	52 ± 10	2.65 ± 0.24	2.62 ± 0.13
	3	Adrenaline (7.5 × 10^{-7} M)	0.31 ± 0.05***	367 ± 7***	1.86 ± 0.16	1.90 ± 0.21*
	3	Dibutyryl cyclic AMP (10^{-3} M	1.74 ± 0.24	122 ± 17*	2.41 ± 0.27	1.89 ± 0.14*
10+10	7	Control	1.96 ± 0.17	46 ± 9	2.97 ± 0.13	3.80 ± 0.38
	7	Adrenaline (7.5 × 10^{-7} M)	0.56 ± 0.08***	153 ± 9***	1.80 ± 0.29**	2.91 ± 0.75
	6	Dibutyryl cyclic AMP (10^{-3} M)	1.11 ± 0.17**	92 ± 6**	2.44 ± 0.12*	3.30 ± 0.38

Values are means ± S E M The experimental conditions are given in Table III At the end of the perfusion period the hearts were instantly frozen in liquid N_2 and weighed

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

diastolic filling Dibutyryl cyclic AMP did not significantly affect the heart rate or peak systolic pressure after 1 and 2 min of perfusion but after 5 min a significant increase in peak systolic pressure was seen The coronary flow was significantly increased after 5 min of perfusion with adrenaline and after 10 min of perfusion in presence of dibutyryl cyclic AMP

A correlation was found for the time of onset of changes in heart function and metabolic parameters (Table III and IV) Adrenaline significantly decreased the content of glycogen ATP and creatine P and increased lactic acid production after 1 min of perfusion At this time an increase in heart rate in the presence of adrenaline was also seen A significant effect of dibutyryl cyclic AMP was seen first after 5 min of perfusion when an increase in lactic acid production and a decrease in creatine P were demonstrated (Table IV) No change in glycogen or ATP content was however demonstrated after this period of perfusion The increase in peak systolic pressure (Table III) in presence of dibutyryl cyclic AMP was also demonstrated after a perfusion period of 5 min After 10 min of perfusion dibutyryl cyclic AMP significantly decreased the glycogen and ATP contents of the heart and increased its lactic acid production (Table IV)

Discussion

Sutherland and co workers (*e g* 1968) have presented evidence indicating that cyclic AMP is a second messenger' mediating various hormonal responses including the positive inotropic and glycogenolytic effects of catecholamines in the heart To establish the mechanism of cyclic AMP as a second messenger for the action of adrenaline in the heart it is desirable to have four criteria satisfied according to these authors 1) adenyl cyclase activity of a broken cell preparation of the heart should be stimulated by adrenaline 2) the level of cyclic AMP in the intact heart should be elevated by adrenaline added to the isolated tissue, 3) drugs that inhibit phosphodiesterase activity should potentiate the effects of adrenaline and increase the level of cyclic AMP in the heart 4) addition of exogenous cyclic AMP should mimic the effects of adrenaline

In case of the effect of adrenaline on the phosphorylase activation to increase glycogenolysis in the heart all four criteria applied to this response have been met with (Haugaard and Hess 1965 Drummond and Duncan 1966) However concerning the positive inotropic response to adrenaline only the first three criteria have been well established (Sutherland *et al* 1968 Robison *et al* 1967) Recently Kukovetz (1968 reported that in some experiments a positive inotropic effect could be observed by the use of dibutyryl cyclic AMP Besides this observation no inotropic effects after addition of cyclic AMP to the isolated heart have been reported

The result of the present study that addition of dibutyryl cyclic AMP to the isolated working rat heart produced a clear cut and concentration dependent inotropic response is in accordance with the observation of Kukovetz (1968) using a non working heart preparation The fourth criterion required by Sutherland and

co-workers to establish that cyclic AMP might be a "second messenger" for the inotropic response to adrenaline has been satisfied. Our results thus strongly support the hypothesis that cyclic AMP in some way is involved with the positive inotropic effect of catecholamines. We think, however, that not even a complete fulfilment of Sutherland's four criteria creates a definite proof that cyclic AMP is a specific cellular mediator of a hormonal effect. Other cellular substances, for example, other cyclic nucleotides, might also be of importance as direct links between the site of cellular receptor—catecholamine interaction and the increase in contractility.

In contrast to the results obtained by Kukovetz (1968), showing a positive chronotropic effect of dibutyryl cyclic AMP no such effect was found in this study. Instead a significant decrease in heart rate with the highest concentration of dibutyryl cyclic AMP (10^{-3} M) could be observed. This decrease in heart rate after addition of dibutyryl cyclic AMP is in agreement with the observations of Øye (1967), who demonstrated a decrease in heart rate in presence of cyclic AMP. The non-cyclic adenine nucleotides and nucleosides have many similar characteristic pharmacological actions including a negative chronotropic effect and an increase in coronary flow (Berne 1963, Øye 1967, Buckley 1970). It is probable that the negative chronotropic effect with dibutyryl cyclic AMP seen in the present experiments, with high concentrations of the nucleotide, is such an unspecific pharmacological effect. The possibility that the decrease in heart rate produced by dibutyryl cyclic AMP in this study should explain the increase in peak systolic pressure is improbable. At a lower concentration of dibutyryl cyclic AMP (10^{-5} M) a positive inotropic effect was seen, when the heart rate was not changed. Furthermore, when hearts were electrically paced to keep the heart rate constant the peak systolic pressure of hearts perfused with the nucleotide was higher than for the control hearts.

The reason why Robison *et al.* (1965) in contrast to our observation could not obtain an inotropic effect of dibutyryl cyclic AMP when added to the perfused working heart is not clear. There is, however, one important difference between the two studies. These authors used hearts of rats weighing 275—325 g, while in our study the rats were weighing 80—90 g. Robison *et al.* (1965) calculated that cyclic AMP at a concentration of 10^{-4} M was destroyed five times faster than it was calculated to entry the cells of the perfused rat heart. The difference between the mentioned studies might be that the permeability of the cell membranes for dibutyryl cyclic AMP is better in hearts of younger rats since it is well known that permeability properties are more favourable in younger animals. Differences in the phosphodiesterase activity between young and old rats could also be of importance.

In short time perfusions of the present study significant metabolic and positive inotropic effects of dibutyryl cyclic AMP were not seen until 5 min after its addition to the perfusate. A significant increase in heart rate and lactate production and a decrease in the heart content of glycogen, ATP and creatine P were demonstrated already after a perfusion period of 1 min in presence of adrenaline. The extremely rapid effects of adrenaline on inotropic and metabolic responses in the heart are well documented (*e.g.* Mayer *et al.* 1967). The delayed effects of dibutyryl cyclic AMP

might again be explained as due to a slow cellular penetration rate of the nucleotide. Another factor could be that the esterase enzyme capable of removing the acyl group from the 2' position of the ribose molecule is rate limiting since it has been demonstrated that the 2'O-monoacyl derivate of the nucleotide is biologically inactive (Posternak *et al.* 1962).

Attempts to demonstrate inotropic and metabolic effects after addition of cyclic AMP to isolated atria or intact hearts have not been successful (Rall and West 1963, De Gubareff and Sleator 1965, Robison *et al.* 1965, Øye 1967). It must therefore be questioned whether the effects of dibutyryl cyclic AMP in the heart in this study could be due to an effect of the dibutyryl derivate *per se* rather than of the cyclic AMP. This seems not very likely since substitution with an acyl group in the 2' position of cycle AMP gives a substance without activity when tested in extracts of liver and heart, suggesting that the activity of an esterase enzyme is necessary to elicit the effects of dibutyryl cyclic AMP (Posternak *et al.* 1962, Henion *et al.* 1967).

Dibutyryl cyclic AMP decreased the glycogen content of the heart and it is probable that this is a reflexion of an increased activity of phosphorylase. Part of this effect could, however, be due to an inhibition of glycogen synthetase since it has been shown that adrenaline and exogenously administered cyclic AMP inhibit glycogen synthetase in liver from mice (De Wulf and Hers 1968). This observation has been confirmed by Bishop and Larner (1969) using a purified glycogen synthetase system from dog liver. It is not likely that the very marked decrease in glycogen content of the hearts perfused with dibutyryl cyclic AMP is due to the increased mechanical performance *per se*, since an increase in work load does not give very large changes in glycogen content of the working rat heart preparation when perfused with high concentrations of glucose (Neely *et al.* 1968).

Propranolol was used in this study to block the effects of catecholamines which could be mobilized from endogenous stores during the perfusion with dibutyryl cyclic AMP. Although propranolol (10^{-5} M) almost completely abolished the cardiac response to adrenaline (7.5×10^{-7} M) it did not reduce the effects of dibutyryl cyclic AMP (5×10^{-5} M). The metabolic and inotropic effects of the nucleotide in this study are not due to activation of adrenergic β receptors by endogenously released catecholamines. When propranolol was used at a higher concentration (3×10^{-6} M) marked negative chronotropic and inotropic effects were observed confirming earlier reports (Blinks 1967, Buckley 1970). Even at this higher concentration of propranolol the effects of dibutyryl cyclic AMP were not reduced and the nucleotide could compensate for the negative inotropic and chronotropic effects of propranolol. This indicates that dibutyryl cyclic AMP can exert both positive inotropic and chronotropic effects. In the experiments with dibutyryl cyclic AMP in absence of propranolol a positive chronotropic effect has not been demonstrated. Thus a positive chronotropic effect of dibutyryl cyclic AMP might be masked under some experimental conditions.

The mechanisms by which catecholamines, cyclic AMP or its dibutyryl derivate enhances myocardial contractility are not known. The interaction between actin and

myosin must be affected in some way and it might be reasonable to expect that catecholamines and the nucleotides increase the cardiac actomyosin ATPase activity However, no direct effects of catecholamines or cyclic AMP on the contractile proteins have been established (Mommaerts *et al* 1963, Katz 1970) It has been postulated that cyclic AMP may act on the excitation contraction mechanism by increasing the amount of calcium ions released during each contraction (Nayler 1967) Recently, the sarcoplasmic reticulum of canine myocardium was reported to contain an adenyl cyclase system, which is sensitive to noradrenaline, and that noradrenaline and cyclic AMP increased the accumulation of calcium in this preparation (Entman *et al* 1969 a, 1969 b) The increased amount of calcium in the sarcotubular system may modulate the strength of myocardial contraction

Recently Langslet and Øye (1970) found that administration of cyclic AMP to the isolated working rat heart at a low temperature can produce increased phosphorylase activity and glycogenolysis without any effect on the mechanical performance of the heart This observation shows that the relationship between metabolic and mechanical effects in the heart is complex and yet unknown in many details Another illustration of the same fact is the observation of Shanfeld *et al* (1969) that N isopropyl methoxamine can block both the increase of cyclic AMP and the increase in phosphorylase activity produced by norepinephrine in the perfused rat heart (Langendorff preparation) without prevention of the inotropic effect of the amine Further studies are therefore necessary to elucidate the relationship between metabolic and mechanical effects of the catecholamines on the heart muscle

Valuable technical assistance was given by Miss Elisabet Johansson

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From the Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden

Histamine and Heparin Release from Isolated Rat Mast Cells Exposed to Compound 48/80

By

STUART A. SLORACH

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Abstract

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The relationship between the release of histamine and heparin from rat mast cells exposed to compound 48/80 *in vitro* has been studied in order to elucidate the mechanism of action of this histamine releasing agent. Heparin is almost exclusively localized to the granule fraction of mast

to compound 48/80 suggest that the most mature granules are released first on treatment of mast cells with this substance.

Mast cells are the most important storage sites for both histamine and heparin in many mammalian tissues (*vide* Riley 1959 and Jorpes 1963). In rat peritonal and pleural mast cells the histamine is stored ionically linked to intracellular granules which consist essentially of a complex formed from heparin and basic protein (*vide* Lagunoff *et al.* 1964, Uvnas, Åborg and Bergendorff 1970).

Uvnas and Thon (1966) proposed that the release of histamine from rat mast cells treated with compound 48/80 *in vitro* involved an initial release of histamine containing granules from the cells followed by an ion exchange between histamine in the extruded granules and cations in the extracellular medium. Subsequent studies from this laboratory have provided further experimental support for this hypothesis (Thon and Uvnas 1967, Fillion, Slorach and Uvnas 1970, Nosal, Slorach and Uvnäs 1970).

Since virtually all the heparin in mast cells is localized to the granule fraction (Lagunoff and Benditt 1963), a study of its release should provide reliable data about granule release If the release of histamine induced by compound 48/80 takes place in the way proposed by Uvnas and Thon the release of histamine and of heparin should be quantitatively related this correlation has been investigated in the present study Furthermore, by using mast cells taken at different times after injecting the rats with $Na_2^{35}SO_4$ and studying the specific activity of the ^{35}S labelled heparin in the released and non released granules after treating the cells with compound 48/80 it was hoped to obtain information about the synthesis storage release cycle of heparin in rat mast cell granules

Methods and Materials

Labelling and isolation of mast cells

Mast cells were isolated from the pleural and peritoneal cavities of male Sprague Dawley rats (b w 350—400 g) injected 2 to 45 days earlier with 1 or 2 mCi $Na_2^{35}SO_4$ as described by Thon and Uvnas (1966)

Separation of heparin from other mast cell constituents

^{35}S labelled mast cells from 6 rats injected 30 days earlier with $Na_2^{35}SO_4$ were dissolved in 2 ml of 0 05 N NaOH, 8 ml of 1 M NaCl was added and the solution was adjusted to pH 7 with hydrochloric acid The solution was then applied to a Dowex 1 X2 column (10×200 mm, 50—100 mesh) prepared in 1 M NaCl The column was eluted (0 5 ml/min 5 ml fractions) with 1 M NaCl until 60 ml of eluate had been collected and then with 2 M NaCl (30 ml) and finally with 4 M NaCl (20 ml) The absorption at 230 nm (guide to protein content), the radioactivity (*vide* Fillion *et al* 1970) and the heparin content (*vide infra*) were measured in aliquots of each fraction Nearly all the protein was found in the 1 M NaCl eluate whereas the heparin and radioactivity were confined to the 2 M NaCl eluate (Fig 1) There was a very similar relation between the radioactivity and heparin contents in each fraction indicating that the ^{35}S in the cells was present in heparin molecules

The recovery of heparin (pig mucous) in the 2 M NaCl eluate after passing through a Dowex column as above was 98—100 per cent Inorganic sulphate ($Na_2^{35}SO_4$) applied to such a column was eluated in the 1 M NaCl fraction

Heparin assay method

Heparin was assayed by the method of Glick von Redlich and Diamant (1967) as modified by Fillion *et al* (1970) The precipitation of Azure A by heparin is markedly affected by the presence of sodium chloride and therefore the concentration of sodium chloride used in the standard solutions was matched with that in the solutions being assayed According to Jaques (1961) rat heparin contains 13 7 per cent sulphur The heparin used to prepare the standard solution in the present experiments contained 9 2 per cent of sulphur and the values obtained in the assays were converted to and are expressed as rat heparin using the factor 0 67

Release studies

The release studies were carreid out on mast cells isolated from rats injected with 1 mCi $Na_2^{35}SO_4$ 2 7 or 16 days earlier or with 2 mCi of the isotope 45 days earlier ^{35}S labelled mast cells from 10—12 rats were incubated (37° C 10 min) with or without compound 48/80 [illegible] Fillion [illegible] were [illegible] in [illegible] ion [illegible] ere [illegible] ked disrupted by the [illegible] and sodium chloride solution added to give a final vol of 10 ml and a sodium chloride concentration of 0 85 per cent wt /vol Histamine was assayed in aliquots of the bulked 350×g supernatants and the disrupted cell suspensions as previously described (Fillion *et al* 1970)

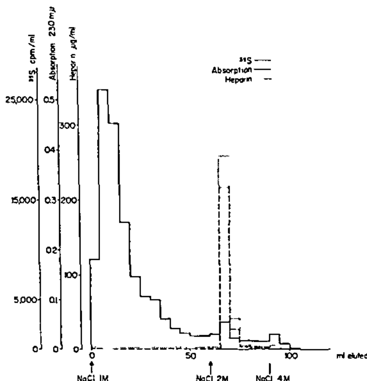


Fig 1 Separation of protein ^{35}S and heparin from mast cells on Dowex 1 X2 The absorption at 230 nm is used as a guide to protein distribution

The supernatants and disrupted cell suspensions were then centrifuged (3000 × g 30 min 4° C) and the resulting supernatants decanted The precipitates were dissolved in 2 ml of 0 05 N NaOH duplicates bulked and 8 ml of 1 M NaCl added before adjusting the solutions to pH 7 with hydrochloric acid Each 3000 × g supernatant and the corresponding solution of the 3000 × g precipitate was then added to a Dowex column (*vide supra*) and the column was eluted as described above The ^{35}S heparin containing fractions were identified from the radioactivity levels in the eluates fractions 2—4 inclusive of the 2 M NaCl eluate contained over 90 per cent of the total radioactivity The fractions were bulked and the content of hepa n nd h d

t on

Materials

Compound 48/80 was a gift from Dr B Hogberg AB Leo Halsingborg Sweden ^{35}S as carrier free sulphate in sterilized neutral isotonic solution was obtained from the Institutt for Atomenergi Kjeller Norway and Azure A from Eastman Organic Chemicals Dept Rochester New York USA Heparin sodium p g mucous) containing 9 2 per cent sulphur was obtained from AB Vitrum Stockholm Sweden

TABLE I Histamine and heparin contents of and histamine, ^{35}S and heparin release from mast cells taken at various intervals after $Na_2\,^{35}SO_4$ injection and exposed to compound 48/80

Labelling[1] time, days		2	7	16	45
Histamine release*	a)	38	48	43	47
	b)	56	65	66	66
^{35}S release*	a)	18	27	28	26
	b)	28	45	44	41
Heparin release*	a)	24	24	22	24
	b)	33	46	38	43
Histamine release* / ^{35}S release*	a)	2 1	1 7	1 5	1 8
	b)	2 0	1 4	1 5	1 6
Histamine release* / Heparin release*	a)	1 6	2 0	1 9	2 0
	b)	1 7	1 4	1 7	1 5
Histamine content[2]		40 6	24 3	26 5	27 9
Heparin content[2]		106 3	74 8	108 5	91 5

[1] Interval between $Na_2\,^{35}SO_4$ injection and cell isolation
[2] *Mean values for all cells in expt , μg per 10^6 cells*
* Percentage of total content, release in untreated cells deducted Compound 48 80 conc a) 0 25 μg/ml b) 2 5 μg ml

Results

Table I shows the release of histamine, ^{35}S and heparin from rat mast cells incubated with compound 48/80 and washed as described in Methods The release from cells not exposed to compound 48/80 but otherwise treated similarly has been deducted in each case The release was calculated from the means of assays carried out in duplicate or triplicate The ratios percentage histamine release/percentage ^{35}S release for cells taken 2 7 or 16 days after isotope injection are similar to those reported by Nosal *et al* (1970) for cells taken 2, 6 and 12 days respectively after isotope injection The ratio percentage histamine release/percentage heparin release varied between 1 6 and 2 0 with 0 25 μg/ml compound 48/80 and between 1 4 and 1 7 with 2 5 μg/ml of the releasing agent

The mean histamine and heparin contents of the mast cells studied were 29 8 μg/10^6 cells and 95 3 μg/10^6 cells respectively The ratio heparin content/histamine content (3 2) was similar to the values found by Benditt, Arase and Roeper (1956) and Bergendorff (to be published) (3 0 and 3 6 respectively)

The specific activities of the S labelled heparin released from and retained by mast cells taken different times after $Na_2\,^{35}SO_4$ injection and exposed to compound 48/80 are shown in Table II The specific activities were calculated from the heparin and radioactivity levels in the bulked 2 M NaCl eluates used for the heparin assays After a labelling time of only two days the non-released heparin had a higher specific activity than the released heparin *i e* there was a preferential labelling of the retained

TABLE II Specific activity of ^{35}S labelled heparin released from and retained by rat mast cells taken at various intervals after $Na_2\,^{35}SO_4$ injection and exposed to compound 48 80

		Specific activity of ^{35}S labelled heparin (cpm/µg)			
Labelling[1] time days		2	7	16	45
Heparin release from cells	a)	117	122	103	75
	b)	120	95	101	64
Heparin retained in cells	a)	149	106	75	59
	b)	147	99	81	60

[1] Interval between $Na_2\,^{35}SO_4$ inj and cell isolation
Compound 48 80 conc a) 0 25 µg/ml b) 2 5 µg/ml

granules No clear pattern emerges from the experiment with cells taken 7 days after isotope injection but with longer labelling times (16 or 45 days) the released heparin had a slightly higher specific activity than that retained in the cells after compound 48/80 treatment Further work is necessary to establish whether or not the differences in heparin specific activity in the granules retained and released by the cells are statistically significant

Discussion

The present experiments show that both histamine and heparin are released when rat mast cells are exposed to compound 48/80 They thus confirm the *in vivo* observations of Riley *et al* (1955) on release of histamine and heparin from rat subcutis As Riley (1959) pointed out, the reason why heparin released from rat mast cells following the administration of compound 48/80 to rats *in vivo* does not reach the blood stream and affect blood coagulability is that the heparin is released in granules which are insoluble in tissue fluids and which are rapidly phagocytosed and digested by nearby fibroblasts (*vide* Higginbotham Dougherty and Jee 1956)

Previous reports from this laboratory (Fillion *et al* 1970 Nosal *et al* 1970) have provided experimental support for the hypothesis advanced by Uvnas and Thon (1966), that the release of histamine containing granules from mast cells is an initial event in histamine release induced by this agent In the present study in which the release of both histamine and heparin have been measured directly correlations between the release of these two substances similar to those reported for histamine release and ^{35}S heparin release by Nosal *et al* (1970) were found Since rat mast cell heparin is almost exclusively localized to the granule fraction (Lagunoff and Benditt 1963) heparin release can be said to reflect granule release If the above mentioned hypothesis of Uvnas and Thon is correct then the ratio percentage histamine release/percentage heparin release should be 1 In the present experiments it was 1 4—1 7 and 1 6—2 0 with compound 48 80 concentrations of 2 5 and 0 25 µg/ml respectively These found ratios are artificially high since in practice it is

impossible to separate all the released granules from the mast cells even with repeated washing (*vide* Fillion *et al* 1970) Bloom and Haegermark (1965) observed swollen less dense (altered) granules in electron micrographs of sectioned compound 48/80 treated mast cells They suggested that such granules which they presumed to be intracellular had released their histamine thus implying that some histamine release could take place without degranulation Similar conclusions were reached by Carlsson and Ritzen (1969) regarding the release of 5 hydroxytryptamine from rat mast cells exposed to compound 48/80 However, by using extracellular markers (lanthanum and hemoglobin) Rohlich Anderson and Uvnas (1971 b) have recently shown that the vacuoles containing altered granules seen in sections of mast cells after exposure to compound 48/80 all communicate with the extracellular space Thus the altered granules observed by Bloom and Haegermark and Carlsson and Ritzen although apparently intracellular are in all probability exocytoplasmic and therefore in contact with the extracellular fluid The electron microscopic studies of Rohlich Anderson and Uvnas (1971 a 1971 b) also provide a plausible explanation of the fact that in the present experiments and those of Nosal *et al* (1970) it has been impossible to achieve a 1 1 ratio between percentage histamine release and percentage granule release The granules which lie in vacuoles in the mast cells exposed to compound 48/80 are exocytoplasmic and will therefore release their histamine since they are in contact with the cation containing extracellular fluid However the granules themselves remain in such intimate contact with the cells that the complete separation of the two is impossible Taking these facts into consideration it is concluded that the present results are consistent with the hypothesis that histamine release from mast cells exposed to compound 48/80 *in vitro* takes place by an extracellular cation exchange between histamine in granules extruded from the cells and cations in the extracellular medium However the results in themselves do not exclude the possibility that a part of the release takes place by a mechanism not involving granule release

The results of the study of the specific activities of ^{35}S labelled heparin in granules released from and retained by mast cells taken after different labelling times like the results of Nosal *et al* (1970) suggest that the first granules to be released from cells exposed to compound 48/80 are the most mature ones This is consistent with the hypothesis of mast cell granule maturation proposed by Combs (1966) and Padawer (1969)—small progranules aggregate to form granules which then undergo a maturation process precursors of heparin being O sulphated and N sulphated in the early stages of this process Presumably it takes several weeks before the newly formed labelled granules reach such positions in the mast cell that they will be released when the cells are exposed to low concentrations of compound 48/80

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From the Laboratory for the Theory of Gymnastics, University of Copenhagen, Denmark

Thermoregulation during Work in Carbon Monoxide Poisoning

By

BODIL NIELSEN

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Abstract

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[illegible]

... the changes in temperature may be a passive reaction to a shift in blood towards the [illegible] in the CO poisoned state

During muscular exercise the deep body temperature increases to plateau levels, in direct proportion to the work load (*e g* Nielsen 1938, Robinson 1949, Wyndham *et al* 1952) The increase in temperature for different types of work, *e g* leg work, arm work, positive and negative work with the legs, shows a relationship with the oxygen uptake rather than with the work load (B Nielsen 1966, 1968, 1969) Studies of I Åstrand (1960) and Saltin and Hermansen (1966) have demonstrated that the plateau level of core temperature is "set" in proportion to the relative work load in the individual, *i e* to the % of maximum oxygen uptake

Greenleaf *et al* (1969) changed the maximum oxygen uptake capacity (max V_{O_2}) in 6 subjects by exposing them to low O_2 tensions in a low pressure chamber The subjects, exercising at the same absolute work load at normal and at low barometric pressures, would be working at a higher relative load in the low pressure experiments They found, however that the acute exposure to low O_2 failed to change the work temperature level This finding is in agreement with previous observations of Asmussen and Nielsen (1947)

In the present study a mild to moderate CO poisoning was used to lower the maximal oxygen consumption The thermoregulatory responses to 1 hr work was compared at two different work loads and 2 levels of CO hypoxaemia

Methods

within $\pm 0{,}025°$ C

The skin temperature (T_s) was measured at 15 locations with a skin thermocouple (B Nielsen

igle

olle

eat

secretion was calculated from the total weight loss by subtracting the respiratory water loss and respiratory weight loss due to the gas exchange The respiratory gas exchange was measured by

was calculated for the last 15 min of the experiment as

$$K = \frac{H - E_t - S}{(\overline{T}_{es} - \overline{T}_s)A_D} \text{ kcal/hr-m}^2\text{-°C}$$

where E_t = the evaporative heat loss in the lungs (kcal/hr)

S = storage of body heat, positive for heat gain

$= (\overline{T}_{es} \times 0\ 65) + (\overline{T}_s \times 0\ 35) \times$ body weight $\times$ specific heat of body (kcal/hr)

A_D = DuBois surface area (m^2)

The lactid acid concentration in the venous blood sampled after 60 minutes of work was determined by an enzymatic method (Biochemica Test Combination TC-B 15972 Boehringer und Soehne, GMBH, Mannheim)

Muscle blood flow was estimated from the clearance of ^{133}Xe dissolved in isotonic saline and injected in m vastus lateralis (Lassen *et al* 1964, Siggaard Andersen and Petersen 1967)

The experiments were performed in a climatic chamber at $20 \pm \frac{1}{2}$ C ambient temperature without forced air movement

Procedure

After the positioning of the esophageal and rectal thermal probes the subject rested for 3 4 hr on a cot He then started breathing through a Krogh metabolism apparatus filled with atmospheric air Oxygen was introduced at 5 min intervals in sufficient amounts to replace the oxygen used by the subject

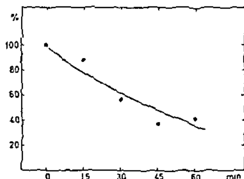


Fig 1

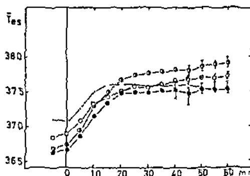


Fig 2

Fig 1 Elimination of CO in percentage of the original concentration in relation to time Work rate 792 kpm/min

Fig 2 Averages of esophageal temperature ($\overline{T}_{es}$) during 60 min of work in

normal condition	(● 6 exper)
25 % CO poisoning	(○ 3 exper)
33 % CO poisoning	(◐ 4 exper)
11,5 % O_2 breathing	(1 exper)

Vertical bars indicate the range

$\overline{T}_o = 20°$ C

Subj JHB Work intensity 601 kpm/min

filled with the required CO ...
absorbe ...
out the ...
were m ...
min and the hea ...
exercise the secon ...
weighing, less than 5 min after the work had ended

In the control experiment the procedure was similar, except that CO breathing and blood sampling were omitted

In two experiments the subjects breathed a gas mixture with 11 5 % oxygen in nitrogen The *mixture was bubbled* through water in a Douglas bag, which was always kept half filled The subject inspired this air mixture through a wide bore respiration valve throughout the whole 60 min work period

The maximal O_2 uptake (max V_{O_2}) was measured during both normal and CO poisoning con*ditions* on a normal Krogh ergometer with saddle After a 7 min warm up at 450 kpm/min the work load was set at a supermaximal work load which would exhaust the subject within 3—5 min

TABLE I

	age yrs	weight kg	height cm	A_D m^2	normal max V_{O_2} l/min	CO max V_{O_2} l/min	O_2 cap vol %	CO vol %	CO vol % in % of total O_2 cap %
JHB	22	70 5	184	1,93	3,65	3,09 2,25	23,7	5,37 7,26	25 33
PJC	26	75,7	178	1,93	3,20	2,42	19,1	5,93	30
*FD	24	77,6	178	1,95	3,78	2,99			26

* Preliminary experiments performed by cand scient Gorm Hylsbjerg as part of his final examination

Fig 3 Equilibrium values of $\overline{T}_{es}$ plotted against O_2 uptake (right) and against relative load (% of max $\dot{V}_{O_2}$) (left)

	normal	25 % CO poisoning	30—33 % CO poisoning
JBH	●	○	◑
PJC	▲		△

Vertical bars indicate the range

and average degrees of CO blocking are also presented

Results

It appeared that during the carbon monoxide hypoxaemia there was a rise in the plateau value of esophageal temperature although the O_2 uptake, heat production and work rate were the same Fig 2 shows the time relationships of esophageal temperature in subject JHB for a work intensity of 601 kpm/min and with 2 levels of CO poisoning, 3 experiments with 24 % and 4 expts with 33 % of the blood oxygen capacity blocked by CO Similar reactions were seen in 2 other subjects In one experiment the subject breathed 11 5 % O_2 for 50 min of exercise The low oxygen tension did not change the level of esophageal temperature, this experiment fell within the range of the normal control experiments The same was seen in 1 expt on a second subject

In Fig 3 the averages of esophageal plateau temperature values are plotted against both the absolute oxygen uptake and the percentage of the maximal oxygen uptake capacity for each subject and condition There was a linear relationship between temperature and the relative load for the two subjects

Averages of the measured temperatures, oxygen uptakes heart rates, sweat rates, and the calculated values of conductance for the 2 principal subjects are presented in Table II

The muscle blood flow in m vastus lateralis was estimated by the ^{133}Xe clearance method in 6 expts in the normal, the CO poisoned and the low oxygen con-

dition The results may indicate that the local muscle flow was greater in the CO experiments than in the normal condition However, due to the few experiments the results were inconclusive

The lactic acid concentration of the venous blood after 60 min of work was determined in 2 normal, 3 CO, and 2 low O_2 experiments After work in the normal condition the concentration was not increased above the resting level 12 mg % for JHB and 7,9 mg % for PJC In the CO experiments it was 66 mg % and 44 mg % for JHB working at 792 kpm/min and 55,4 mg % for PJC at 601 kpm/min In the low oxygen experiments blood lactate was also somewhat increased 19,5 mg % for JHB and 30,9 mg % for PJC both at 601 kpm/min

Discussion

The equilibrium body temperature during exercise was found to be higher when the stress on the subject was increased by decreasing his maximal oxygen intake capacity by blocking his oxygen transport system with carbon monoxide The temperature rise was proportional with the decrease in max $\dot{V}_{O_2}$ This finding is in agreement with the results of I Åstrand (1960) and Saltin and Hermansen (1966) where subjects of widely different maximal O_2 capacities obtained the same esophageal temperature when working at the same percentage of their maximal capacity Clasing and Laumann (1968) similarly found that subjects in different states of training obtained the same rectal temperature, 38° C, at a pulse rate of 130

However earlier experiments in which the maximal oxygen uptake capacities were lowered to the same degree by having subjects work at the same load at sea level and at simulated altitude (Asmussen and Nielsen 1947, Greenlaef *et al* 1969)

TABLE II Averages of steady state values of heart rate pulmonary ventilation oxygen uptake and 3 measurements between 35th and 60th min Sw the total sweat loss in the work period are presented in parenthesis

subject	work rate kpm min		heart rate	V_E	V_{O_2}	% V_{O_2}max	RQ
JHB	792	normal n 6	111 (102—118)	48 7 (45 2—54 6)	1 87 (1 77—2 06)	51	0 88 (0 83—0 94)
		25 % CO n 5	149 (144—158)	59 9 (57 0—62 7)	1 94 (1 86—2 06)	63	0 94 (0 90—1 09)
JHB	601	normal n 6	93 (90— 98)	38 0 (34 6—41 4)	1 45 (1 39—1 53)	40	0 90 (0 87—0 94)
		25 % CO n 3	120 (119—122)	38 5 (35 0—43 6)	1 46 (1 43—1 51)	47	0 93 (0 88—1 02)
		33 % CO n = 4	137 133—140)	43 7 (37 5—48 4)	1 50 (1 40—1 60)	68	0 94 (0 83—1 00)
PJC	601	normal n 4	125 (120—128)	32 4 (31 5—33 4)	1 50 (1 45—1 55)	47	0 88 (0 87—0 92)
		30 % CO n = 4	170 168—172)	46 8 (44 5—52 9)	1 57 (1 54—1 61	65	0 95 (0 88—1 02)

showed no effect on the temperature setting The exercise temperature level was *found to be unchanged at acute exposure to altitude The two experiments with low* O_2 breathing one on each of 2 subjects in the present study, are in agreement with the altitude studies

The most striking difference between the normal and the CO poisoned state is the very low venous oxygen tensions obtained in the latter situation The arterial oxygen tension is normal in the CO experiments, but the arterial blood only contains 67—75 % of its normal volume of oxygen Further, the affinity of O_2 to the hemoglobin which is not bound to CO, is increased In other words the oxygen dissociation curve is shifted to the left in the presence of CO so that extraction of oxygen from the blood requires a very low tissue tension (Douglas *et al* 1912 Stadie and Martin 1925 Roughton and Darling 1944) Finally, there is no compensatory increase in the cardiac output as when breathing low O_2 mixtures (Asmussen and Chiodi 1941, Chiodi *et al* 1941, Klausen *et al* 1968) The high blood lactate values in the present CO experiments confirm the high level of anaerobicity

The effect on the temperature regulating mechanism of the reduction in aerobic power produced by the CO poisoning might be a direct effect on the centers in the brain The low O_2 tension might change the activity of some of the neurons in the temperature center and by that the body temperature This assumption cannot be ruled out nor confirmed by the present experiments

The low O_2 tensions on the venous side of the circulation, which result from the CO blocking might also be thought to act on peripheral venous chemoreceptors *e g*, in the working muscles, in the veins draining the working muscles in the right heart, *or in the pulmonary arteries In the normal condition mixed venous O_2 tension and*

RQ $\overline{T}_{es}$ and $\overline{T}_{re}$ are means of measurements between 50th and 60th min of work $\overline{T}_s$ the mean of while K is calculated for the 45th to 60th min n number of experiments The range of the values

$\overline{T}_{es}$	$\overline{T}_{re}$	$\overline{T}_s$	S_w	K	$\overline{T}_o$
37.72	*37.86*	*31.44*	*286*	*32.0*	*20.9*
(37.58—37.88)	(37.58—37.99)	(31.09—31.59)	(200—370)	(28.8 36.6)	(20.2—21.6)
38.11	38.14	30.40	373	26.4	21.3
(37.95—38.20)	(38.00—38.29)	(29.37—31.03)	(336—416)	(23.4—31.8)	(20.3—23.5)
37.54	37.68	30.69	147	22.4	20.5
(37.47—37.63)	(37.47—37.63)	(30.53—31.05)	(116—168)	(19.9—26.1)	(29.2 21.0)
37.71	37.64	29.70	169	19.2	20.7
(37.68—37.72	(37.54 37.71)	(29.67 29.82	(134—192)	(18.4—20.4	(20.6—20.7)
37.88	37.89	29.89	218	0.3	20.5
(37.83—37.97	*(37.81—38.01)*	*(29.64—30.22)*	*(184—246)*	*(18.9 21.7)*	*(20.3—20.9)*
37.58	37.6	29.09	272	20.0	20.5
(37.42 37.72	(37.56—37.71	28.79—29.39	(199—416	(19 20.5	(20.3—20.8)
37.89	37.89	28.34	347	18.9	20.4
(37.75—38.03	(37.58—38.14	(27.49—28.80	(307—412	(17.9 20.2)	(20,2—21.0)

probably also muscle O_2 tension is inversely related to the metabolic rate The relationship between O_2 uptake and body temperature during work (B Nielsen 1966, 1969) might thus depend on signals arising in venous chemoreceptors Correspondingly, the excessive lowering of the venous tensions in the CO experiments might produce the higher body temperatures in this situation In low O_2-breathing and at altitude on the other hand, the low arterial O_2-tension stimulates the chemoreceptors in the carotid and aortic bodies This induces compensatory increases in pulmonary ventilation and cardiac output Further, the O_2 dissociation curve is less steep, so that the venous O_2-tensions remain at nearly the same values as in normoxia Against the assumption of the venous P_{O_2} as the active stimulus for the thermoregulatory center speaks the fact, that at the same oxygen uptake, the cardiac output is the same for trained and untrained individuals in the normal condition (Ekelund and Holmgren 1967) Therefore, at the same heart rate and relative load, an untrained subject has a lower O_2 uptake and cardiac output, and hence a smaller (a v) O_2diff and a higher mixed venous P_{O_2} than a trained subject But the body temperature will be the same

The observed differences between the plateau level of core temperature in the normal and CO poisoned states might also be thought to be related to hemodynamic factors Since heat dissipation is dependent on the heat transported by the blood to the skin, changes in peripheral circulation and distribution of the blood might be the cause of the temperature rise The cardiac output during work is nearly normal in CO-hypoxemia (Asmussen and Chiodi 1941, Chiodi *et al* 1941, Klausen *et al* 1968)

CO-hypoxaemia apparently causes peripheral vasodilation with pooling of blood in resting organs, *e g*, liver and kidney (Asmussen and Erreboe-Knudsen 1943, Pauli *et al* 1968) The amount of blood available for heat transport, correspondingly, seems to be decreased in the present CO experiments as compared to normal Skin blood flow values, expressed as K, the conductance of the peripheral tissues, are shown in Table II The mean skin temperature in the CO-experiments is on an average one degree lower than normal (Table II) The low skin temperature results in a diminished heat loss by convection plus radiation (C+R) which is compensated by a greater evaporative heat loss This shift in avenues for heat loss, and the increase in body temperature, may thus be explained as primarily a haemodynamic effect of the CO-hypoxaemia In spite of the pooling of blood in the splanchnic organs caused by the low tissue O_2-tension the local blood flow through the muscles may have increased as indicated by the increased ^{133}Xe clearance This would further diminish the amount of blood available for the skin circulation The diminished skin blood flow carries less heat to the skin, which becomes cooler and gives off less heat by convection and radiation Consequently the central temperature must rise The increased gradient promotes the heat transfer from core to skin, both the conductive and the convective, as each l blood will transport more heat *Further, the higher core* temperature seems to stimulate sweating, since the heat balance is maintained with a higher evaporative heat loss and a reduced (C+R) heat loss compared to normal

(Table II) The difference in reactions of deep body temperature in CO hypoxaemia and hypoxic hypoxaemia could thus be explained by the centrally controlled compensatory vasoconstrictions elicited from arterial chemoreceptors in the latter situation (Stenberg, Ekblom and Messin, 1966) The higher cardiac output secures an adequate blood flow for heat transport to the skin without the need of a central temperature increase

If, on the other hand, the central temperature rise in the CO experiments were a regulatory adjustment and not a passive result of the haemodynamic changes, the observed change in sweat rate and skin temperature may be caused by a stimulation of the sweating mechanism by the increased deep temperature The increased evaporative cooling of the skin increases the core surface gradient and, in this manner diminishes the need of skin blood flow

The reason for the close relationship found between core temperature and the relative load in the CO experiments and in normal individuals by interindividual comparisons remains obscure Most likely it is due to differences in the distribution of the cardiac output in trained and untrained individuals According to the findings of several authors an untrained subject distributes a greater part of his total cardiac output to the muscles and less to the splanchnic organs and to the skin (Grimby *et al* *1967*, Rowell *et al* *1964*, Rowell *1969*, *Clausen and Trap Jensen 1970*, *Clausen and* Lassen 1970) This may necessitate that the central temperature in the untrained increases to a higher level as discussed above with reference to the CO hypoxaemic state

Both trained and untrained subjects have regulated body temperature during work, within a wide range of external temperatures, necessitating a more than sixfold variation in skin blood flow This thermoregulatory adjustment of skin circulation may, however, be superimposed on another mechanism, *i e*, the above mentioned differences in the distribution of cardiac output Such an assumption may explain the closer relationship observed when body temperature is compared to relative rather than to absolute work load

The Xenon clearance measurements were performed by dr F Bonde Petersen (Dept of Medical Physiology A) and dr H G Knuttgen (Boston University) revised the English text which is thankfully acknowledged

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From the Departments of Histology and Physiology, Karolinska Institutet, Stockholm, Sweden

Noradrenaline Uptake and Fluorescence Histochemistry in Bovine Splenic Nerves

By

BERTIL HAMBERGER, TORBJORN MALMFORS and LENNART STJÄRNE

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Abstract

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α-methyl-noradrenaline resulted in a strong concentration dependent increase of the fluorescence intensity even in the presence of reserpine. Under the experimental conditions the tissue/medium ratio was found to be about 60 using 10^{-6} M racemic ^3H-noradrenaline in the incubation medium. It is concluded that the axons of the bovine splenic nerve trunk possess a reserpine resistant mechanism for concentrating noradrenaline which is qualitatively similar to but quantitatively considerably less efficient than that previously observed in nerve terminals in other tissues and species.

The nerve fibres of the bovine splenic nerve trunk belong almost exclusively to the C group and mainly represent postganglionic sympathetic axons (Hillarp 1960). The high noradrenaline (NA) content in this tissue has made it a suitable source for preparations of the specific particles (nerve granules) storing a major proportion of the NA in the axon (Euler 1958). The intact trunk has also been used for studies of mechanisms involved in the neuronal biosynthesis of NA (Roth, Stjarne and Euler 1968) as well as of the uptake of exogenous NA from the incubation medium *in vitro* (Stjarne *et al.* 1970). Although the preparation was found to take up NA from the medium, its concentrating ability, as judged by the techniques used in this latter study, was relatively low, considering that it consists almost entirely of sympathetic non-terminal axons. This finding was in apparent contrast to fluorescence micro-

scopic observations and isotope experiments on the rat iris, which showed a marked NA concentrating ability of the adrenergic nerve terminal and non-terminal axons (Malmfors 1965, Olson *et al* 1968, Jonsson *et al* 1969) The discrepancy might be due to species and organ differences, or to differences in the techniques used

In view of this, the present study was initiated, with the aim to study the NA uptake in the bovine splenic nerve trunk *in vitro* and to compare the results of fluorescence microscopy with the tissue/medium ratio for ^{3}H-NA in the same pieces of nerve trunk

Material and Methods

animals
ted free
ll pieces,
15 min
M) or
) NA or
α methyl NA were added to final concentrations of 0 1 (6×10^{-7} M) or 1 (6×10^{-6} M) μg/ml and the incubation was continued for 30 min In some cases the tissue pieces were then transferred to amine free buffer and further incubated at 37° C for 10 or 30 min Incubations were also carried out in other media potassium phosphate, 0 13 M pH 7 4, choline Krebs buffer (sodium replaced by choline), or sodium free Krebs Ringer bicarbonate buffer (sodium replaced by potassium) After the incubations, some pieces were frozen, freeze-dried and prepared for fluorescence microscopy according to Hamberger (1967) while others were spread as whole mounts on object slides and prepared for fluorescence microscopy according to Olson (1969) In some preparations the formaldehyde treatment was omitted

Experiments were also performed with combined isotope and fluorescence microscopical studies according to Olson *et al* (1968) Pieces about 2 cm long, weighing between 0 5—1 5 mg were incubated in 5 ml Krebs Ringer bicarbonate buffer containing 1 1 × 10^{-6} M ^{3}H NA (racemic form about 10 C/mmole) at 37° C for 30 min The tissue pieces were then blotted on filter paper weighed spread on glass slides treated with formaldehyde gas and examined in the fluorescence microscope Finally the nerve spreads were scraped off the glass slides and dissolved in Soluene® (Packard) The radioactivity was determined by liquid scintillation counting The uptake ratio $\left(\frac{\text{dpm/1 mg tissue}}{\text{dpm/1 μl medium}}\right)$ was determined In certain preparations only part of the nerves had accumulated NA The proportion of non fluorescent nerves was estimated in the microscope and was corrected for in the calculations

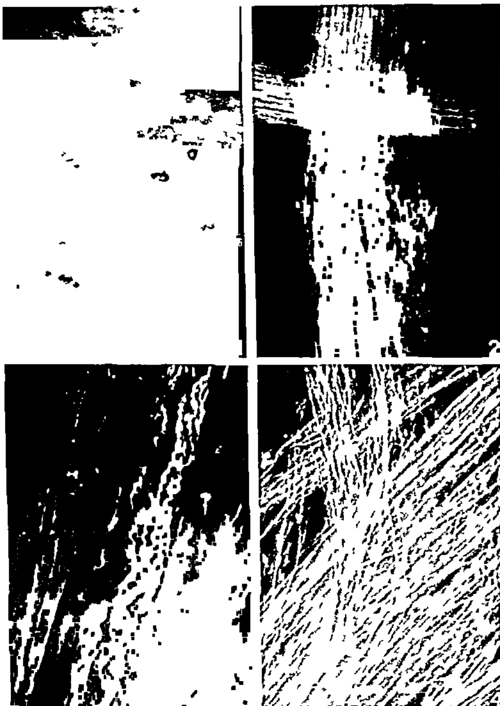
Results

When formaldehyde treatment was omitted only very faint background fluorescence could be detected in the splenic nerve tissue pieces After formaldehyde treatment most fibres in the nerve showed a weak specific green fluorescence, usually with a smooth appearance However a small number of fibres exhibited an intense fluores-

Fig 1 Spread preparation of normal bovine splenic nerve Bundles of fibres exhibit a weak formaldehyde induced specific fluorescence A few nerve fibres have a strong fluorescence and beaded appearance Some cells with strong green fluorescence are also found 120×

Fig 2 Spread preparation of normal bovine splenic nerve incubated in amine free buffer for 30 min A strong accumulation of fluorescent material is seen adjacent to the compression 120×

Fig 3 Longitudinal section 10 μ) from bovine splenic nerve incubated in α methyl NA (0 1 μg/ml) for 30 min Only part of the axons have accumulated α methyl NA and show increased fluorescence 120×



F g 4 Spread preparat on ot bo ine splen c nerve After preinci bation with reserp i 10^{-6} M the nerve wa ncuba ed w h meth l NA 0 1 g/ml) for 30 m n The nerve f br show an ncreased flu e cence ntens t due to uptake of α meth l NA (*cf* F g 1) 120×

cence with a beaded appearance (Fig 1) Scattered cells with a strong green fluorescence in the cytoplasm could be seen in most of the pieces The fluorescence in these cells had an uneven distribution These cells presumably correspond to those earlier shown to contain dopamine (Falck *et al* 1959)

The general appearance of the fluorescence was essentially the same, regardless of whether the tissue had been incubated in an amine free medium or prepared *without previous incubation, 0 5, 2 5 or 5 hrs after death* However, after incubation in amine-free medium a strong accumulation of fluorescent material was often observed close to the cut ends of the nerve fibre pieces and also adjacent to nerve areas which had been accidentally compressed during the preparation of the nerves In the latter case the fluorescence intensity was stronger on one than on the other side of the compressed area (Fig 2)

Incubation with NA at a concentration of 0 1 or 1 μg/ml produced a moderate or strong fluorescence intensity, respectively Incubation with NA in the presence of the monoamine oxidase inhibitors nialamide or harmine, or incubation with α methyl NA in the same concentrations without these drugs, resulted in a strong or intense fluorescence respectively (Fig 3) Subsequent incubation in amine free medium lowered the fluorescence intensity in all groups, but the differences between tissues incubated with and without MAO inhibition were still apparent After incubation with α methyl NA particularly a considerable number of fibres showed an uneven intensity in fluorescence and appeared beaded In the tip of the cut ends no fluorescence was seen

Preincubation with reserpine (10 M) did not prevent the increase in fluorescence intensity of the nerves caused by α methyl NA (Fig 4) However, in the presence of protriptyline (10 M) no increase in fluorescence intensity was observed after incubation with α methyl NA, compared to controls incubated in amine free medium (Fig 5)

No increase in fluorescence intensity was observed when the incubation with α methyl NA (0 1 or 1 μg/ml) was performed in isotonic potassium phosphate (Fig 6), in sodium free Krebs Ringer medium (sodium replaced by potassium) or in sodium free choline Krebs Ringer medium (sodium replaced by choline) The tissue/medium ratio (as defined in material and methods) was found to be 63 ± 10 6 (mean ± s e m from 6 experiments) when using a medium concentration of 1 1 × 10^{6} M ^{3}H NA (see Fig 7—8)

Fig 5 Spread preparation of bovine splenic nerve After preincubation with protriptyline (10 M) the nerve was incubated in α methyl NA (0 1 μg/ml) for 30 min The nerve fibres show about the same fluorescence intensity as in untreated preparations (*cf* Fig 1) indicating that no α methyl NA has been taken up and accumulated in the fibres 120×

Fig 6 Spread preparation of bovine splenic nerve The nerve was incubated in *isotonic* potassium phosphate for 30 min containing α methyl NA (1 μg/ml) No accumulation of α methyl NA can be seen in the nerve fibres A few strongly fluorescent fibres and strongly fluorescent cells can be seen as in Fig 1 120×

Fig 7 Spread preparation of bovine splenic nerve The nerve was incubated in an amine free solution for 30 min 190×

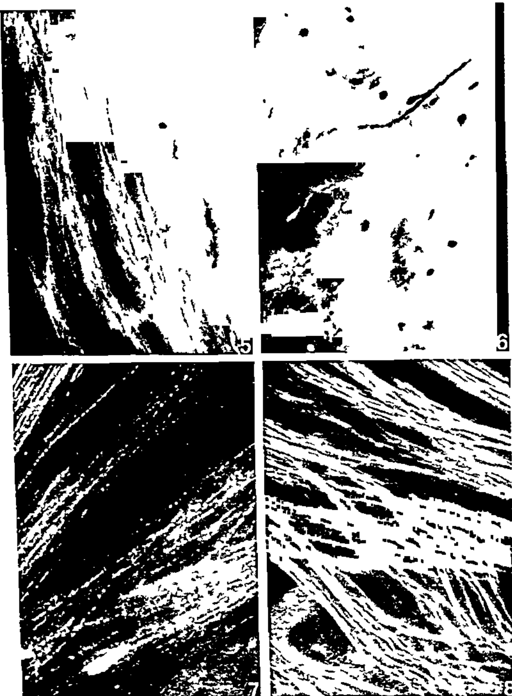


Fig 8 Spread preparation of bovine splenic nerve The nerve was incubated in ^3H NA (1 1 × 10^{-6} M) for 30 min 190 ×

cence with a beaded appearance (Fig 1) Scattered cells with a strong green fluorescence in the cytoplasm could be seen in most of the pieces The fluorescence in these cells had an uneven distribution These cells presumably correspond to those earlier shown to contain dopamine (Falck *et al* 1959)

The general appearance of the fluorescence was essentially the same regardless of whether the tissue had been incubated in an amine free medium or prepared without previous incubation, 0 5, 2 5 or 5 hrs after death However after incubation in amine free medium a strong accumulation of fluorescent material was often observed close to the cut ends of the nerve fibre pieces and also adjacent to nerve areas which had been accidentally compressed during the preparation of the nerves In the latter case the fluorescence intensity was stronger on one than on the other side of the compressed area (Fig 2)

Incubation with NA at a concentration of 0 1 or 1 μg/ml produced a moderate or strong fluorescence intensity, respectively Incubation with NA in the presence of the monoamine oxidase inhibitors nialamide or harmine, or incubation with *α* methyl NA in the same concentrations without these drugs, resulted in a strong or intense fluorescence respectively (Fig 3) Subsequent incubation in amine free medium lowered the fluorescence intensity in all groups but the differences between tissues incubated with and without MAO inhibition were still apparent After incubation with *α* methyl NA particularly a considerable number of fibres showed an uneven intensity in fluorescence and appeared beaded In the tip of the cut ends no fluorescence was seen

Preincubation with reserpine (10^{-5} M) did not prevent the increase in fluorescence intensity of the nerves caused by *α* methyl NA (Fig 4) However in the presence of protriptyline (10^{-5} M) no increase in fluorescence intensity was observed after incubation with *α* methyl NA compared to controls incubated in amine free medium (Fig 5)

No increase in fluorescence intensity was observed when the incubation with *α* methyl NA (0 1 or 1 μg/ml) was performed in isotonic potassium phosphate (Fig 6) in sodium free Krebs Ringer medium (sodium replaced by potassium) or in sodium free choline Krebs Ringer medium (sodium replaced by choline) The tissue/medium ratio (as defined in material and methods) was found to be 63 ± 10 6 (mean ± s e m from 6 experiments) when using a medium concentration of 1 1 × 10^{-6} M ^{3}H NA (see Fig 7—8)

Fig 5 Spread preparation of bovine splenic nerve After preincubation with protriptyline (10^{-5} M) the nerve was incubated in *α* methyl NA (0 1 μg/ml) for 30 min The nerve fibres show about the same fluorescence intensity as in untreated preparations (*cf* Fig 1) indicating that no *α* methyl NA has been taken up and accumulated in the fibres 120 ×

Fig 6 Spread preparation of bovine splenic nerve The nerve was incubated in isotonic potassium phosphate for 30 min containing *α* methyl NA (*1 μg/ml*) No accumulation of *α* methyl NA can be seen in the nerve fibres A few strongly fluorescent fibres and strongly fluorescent cells can be seen as in Fig 1 120 ×

Fig 7 Spread preparation of bovine splenic nerve The nerve was incubated in an amine free solution for 30 min 190 ×

trunk had concentrated the exogenous NA 100 to 1000-fold Quantitative evaluation of the concentrating ability by the radiometric technique showed a tissue/medium ratio of about 60 Assuming that sympathetic axons make up about 50 % of the total tissue mass in the nerve trunk (*cf* Stjarne *et al* 1970), this implies that the axons had concentrated the exogenous amine about 120-fold This brings the radiometric results within the range of the estimate based on fluorescence microscopy Disregarding the possibility of exchange of endogenous for exogenous NA, and also the fact that racemic tracer was used the present results indicate that the uptake of exogenous NA in the axons corresponded to about 22 μg/g Since the endogenous NA content of whole bovine splenic nerve trunk has been estimated to 10—15 μg/g (Euler 1948) and therefore about 20—30 μg/g in the axons, it follows that the total NA concentration was approximately doubled by uptake of exogenous amine

The average tissue/medium ratio obtained in the present study, corrected for areas observed to show poor uptake, is about 10 times higher than the uncorrected value previously reported for bovine splenic nerve trunk (Stjärne *et al* 1970) The discrepancy appears to be partly due to differences in technique and method of presentation In the previous study the pieces of nerve trunk used for incubation were mostly about 1—2 mm in diameter and only a few mm in length, and correction for uneven uptake could not be made In view of the unexpectedly low efficiency of uptake observed the possibility was discussed that the incubation conditions might have been non-optimal In the present study this was shown to be the case The outermost tip was found to be nearly devoid of fluorescence probably reflecting severe depression of the uptake capacity due to mechanical damage Thus much longer pieces of nerve trunk, of much finer caliber were used in the present radiometric study, and under these improved conditions the tissue/medium ratio obtained increased considerably The less satisfactory experimental conditions used in the previous study (Stjarne *et al* 1970) may explain the moderate inhibitory effects of DMI, and of replacement of sodium with choline of even potassium In the present study the inhibitory effects of protriptyline and of replacement of sodium were very strong

It may be concluded that the axons of the bovine splenic nerve trunk possess a reserpine resistant and therefore presumably granule independent mechanism for concentration of extracellular NA with the same properties qualitatively as those previously observed in non terminal and terminal axons in tissues such as the rat iris (Jonsson *et al* 1969) However while the accumulation of exogenous NA in the rat iris axons on incubation with 10^{-6} M NA under similar conditions was calculated to represent an about 15 000 fold increase in NA concentration over that in the medium the estimated tissue/medium ratio in the axons of bovine splenic nerve trunk was several orders of magnitude below this Treatment of rats with reserpine at a dose known to strongly inhibit uptake of exogenous NA in nerve granules only reduced the V_{max} of the mechanism for neuronal concentration of exogenous NA by 50 % (Jonsson *et al* 1969 The lower concentrating ability of the bovine nerve trunk can thus probably not be explained in terms of scarsity of

nerve granules From earlier (Stjarne *et al* 1970) and the present results it may be concluded that NA is taken up with lower efficiency in the non terminal axons of bovine splenic nerve trunk than in nerve terminals

The investigation was supported by grants from the Swedish Medical Research Council (B71 14X 711 06B B71 14X 2330 04 B71 14X 2479 04B) For generous supports with drugs we are indebted to Swedish Ciba (reserpine) Swedish Pfizer (nialamide) and Merck Sharp and Dohme (protriptyline)

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mechanisms in sympathetic nerve trunk

Acta physiol. scand. 1971. 82. 115—122

From the Department of Physiology, Gymnastik och idrottshögskolan, Stockholm, Sweden

Oxygen Deficit and Muscle Metabolites in Intermittent Exercise

By

J Karlsson and B Saltin

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Abstract

Karlsson, J and B Saltin *Oxygen deficit and muscle metabolites in intermittent exercise* Acta physiol scand 1971 *82* 115—122

the one best related to the point of exhaustion

Blood lactate concentration is often used as an index of anaerobic work and to calculate the amount of lactate which has been produced (Margaria, Edwards and Dill 1933, Margaria 1967) In recent experiments (Hermansen 1969) it was found that blood lactate values steadily increase during heavy intermittent work and may reach values up to 30 mmoles $\times l^{-1}$ at the end of the interval work period

Important questions are whether muscle lactate concentration also increases gradually in step with changes in blood lactate concentration or if a certain critical value, corresponding to that of exhaustion, is attained earlier Moreover, one might ask in which way muscle and blood lactate are related to the oxygen deficit attained during each burst of activity

The purpose of this study was, thus, to investigate the increase in muscle lactate concentration during repeated, bursts of very heavy exercise on a bicycle ergometer in order to evaluate the relationship of muscle lactate concentration to blood lactate

TABLE I Pertinent anthropological and circulatory mean data

Age year	Height cm	Weight kg	Maximal oxygen uptake $l \times min^{-1}$
23 (22—24)	175 (172—180)	69 (65—72)	4 0 (3 8—4 2)

concentration as exhaustion approached Further, since oxygen uptake was also measured the possible relationship between oxygen deficit and the different biochemical variables could be analysed

Subjects and Methods

Subjects were physical education students (n = 3) and all three were familiar with the experimental procedure Pertinent data on the subjects are presented in Table I None of the subjects had been especially trained for sprint events

Oxygen uptake was determined by collecting expired air in Douglas bags Gas analyses were performed with a Haldane technique Blood lactate was determined with an enzymatic method (Scholz *et al* 1959) Muscle glycogen G 6 P and lactate were determined in addition to ATP CP and ADP The enzymatic methods used for these measurements are based on Lowry *et al* (1964) as modified by Karlsson, Diamant and Saltin (1970)

Exercise was performed on a mechanically braked bicycle ergometer Each subject's maximal oxygen uptake was determined using the levelling off criterion, prior to the principal experiments

The subjects came to the laboratory in the morning after a light breakfast and rested in a supine position for one hour before the exercise started During the end of that time resting values for $\dot{V}_{O_2}$ blood lactate and muscle metabolites were obtained No warming up was used prior to the tests

The experiment consisted of 1 min work averaging 2800 kpm × min^{-1} followed by 5 min of rest sitting on the bicycle This was repeated 5 times The experimental procedure is illustrated in Fig 1 and the amount of work performed in the experiment is given in Table II

Expired air was collected throughout the whole procedure The collection period was so divided that oxygen taken up both during each exercise period and during rest periods was determined Blood samples for lactate determination were repeatedly taken from a prewarmed finger tip and the blood was immediately deproteinized in cold perchloric acid The biopsies for metabolite determinations were taken in the lateral portion of the quadriceps muscle (Bergstrom 1967) One biopsy was taken with subjects at rest before the start of exercise and three more biopsies were taken during the interval after the first third and fifth work periods respectively Two further biopsies were obtained at 30 and 60 min during the recovery period Each muscle sample was immediately frozen (within 3—5 sec) in liquid nitrogen and stored at −70° C until analysed

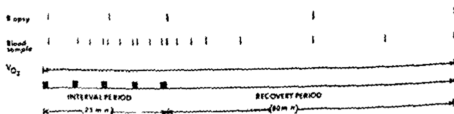


Fig 1 A schematic drawing of the experimental design The black bars denote exercise and the vertical arrows [illegible] when a measurement was *performed*

TABLE II Mean values for different variables during and after the interval period

	I	II	III	IV	V
Work duration min	1 00	0 99	0 93	0 92	0 83
Work load (kpm × min⁻¹)	*2800*	*2910*	*2710*	*2720*	*2760*
Total work kpm	2800	2880	2520	2500	2290
O_2 uptake l	2 8	3 3	2 7	2 8	2 6
O_2 deficit l	3 8	3 5	3 2	3 2	2 9
Recovery O_2 uptake l	6 3	6 3	6 6	6 9	33 1[1]

60 min of recovery after the last work period), based on resting oxygen uptake determined before the interval period started

Results

Oxygen deficit

There is a time lag in the acceleration of oxygen uptake at the onset of exercise As a result of this circumstance and the fact that the work load was supramaximal (Åstrand and Saltin 1961, Karlsson and Saltin 1970), energy from anaerobic processes was quantitatively important in each burst of activity (Fig 2) During the first work period the calculated mean oxygen deficit amounted to 3 8 liters with a slight gradual decrease in oxygen deficit during the remaining work periods (Table II) *To a minor extent this was due to a somewhat larger amount of oxygen*

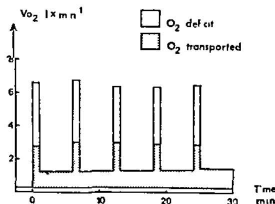


Fig 2 A comparison is shown between the determined amount of oxygen taken up and the oxygen demand for a complete aerobic energy delivery In the calculation of this theoretical value a mechanical efficiency of 22 5 per cent was used The discrepancy between the theoretical value and the oxygen uptake is defined as the oxygen deficit

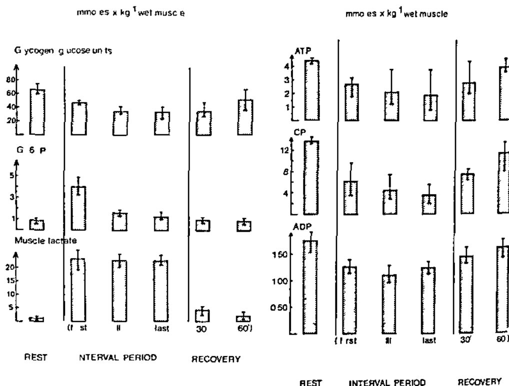


Fig 3

Fig 4

Fig 3 Glycogen G 6 P and lactate concentrations in mmoles × kg ¹ wet muscle in tissue specimens taken from the lateral portion of the quadriceps muscle before (left) during the intermittent exercise period (centre) and during one hour of recovery (right) presented as means (bars and range Biopsies during the interval period were taken immediately after an exercise period Information in parentheses denote the exercise period after which or at which time during the recovery the biopsy was taken

Fig 4 ATP CP and ADP concentrations in the muscle tissue before during and after intermittent exercise presented as in Fig 3 Determinations were made on the same muscle specimens as in Fig 3

transported during each work period but was mainly due to a shorter work time and/or slower pedalling rate as the subjects became exhausted The total oxygen deficit averaged 16 6 (15 0—16 8) liters

Biochemical changes in muscle tissue and in blood

Mean values for ATP CP ADP glycogen, G 6-P and lactate concentrations in the muscle are given in Fig 3 and 4 Rest values for ATP ranged from 4 2—4 6 mmoles × kg ¹ wet muscle The reduction in ATP concentration tended to be more pronounced after the third and fifth activity burst as compared to the first The mean value ob-

tained after the last burst was 1 9 mmoles×kg $^{-1}$ wet muscle ATP values increased during the recovery period but ATP concentration even after 60 minutes of recovery, tended to be lower than the rest value (4 0 compared to 4 4 mmoles×kg $^{-1}$ wet muscle respectively)

The breakdown of ATP was not associated with an elevation in muscle ADP concentration On the contrary, ADP concentrations were found in all subjects to have diminished in the biopsy specimens obtained immediately after the activity bursts ADP concentrations averaged 1 26 mmoles×kg $^{-1}$ wet muscle after the last burst as compared to 1 76 at rest During the 60 min of recovery ADP concentrations increased to rest levels

The rest values for CP ranged from 13 2 to 14 4 mmoles×kg $^{-1}$ wet muscle The lowest CP concentration found after the last burst of activity as was the case for ATP, averaged 3 6 mmoles×kg $^{-1}$ wet muscle During the final recovery period CP values increased faster than the ATP concentration and were essentially the same after 60 min of recovery as before exercise

Total glycogen depletion amounted to 31 mmoles of glucose units×kg $^{-1}$ wet muscle Glycogen breakdown was more pronounced after the first than after the second and third bursts of activity and depletion seemed to be even less during the fourth and fifth periods Complete depletion of muscle glycogen stores was not observed in any of the subjects

The peak value for G 6-P was found after the first burst and increased from 0 8 mmoles×kg $^{-1}$ wet muscle in the resting muscle to 4 2 mmoles×kg $^{-1}$ wet muscle The G 6-P concentration in the working muscle then gradually decreased and averaged 1 3 mmoles×kg $^{-1}$ wet muscle after the last burst of activity After 1 hr of recovery, normal resting values were obtained

Muscle lactate concentration had already reached its highest value after the first burst of activity in two of the subjects (Fig 5) In the third subject only a very minor additional increase was observed after the third and fifth activity periods Mean concentrations after the first third and fifth activity burst ranged from 22 5 to 23 2 mmoles×kg $^{-1}$ wet muscle

In contrast to muscle lactate concentration blood lactate values gradually increased in all three subjects and a plateau was only reached after about 15—20 min Values higher than 20 mmoles×l $^{-1}$ were not observed in any of the subjects

Oxygen debt and heart rate

Oxygen uptake (exceeding pre exercise rest oxygen uptake) during the recovery period including both rest periods in the interval period and the first hour of rest after the last work period averaged 30 2 (26 5—32 9) liters The ratio between the calculated oxygen deficit and the recovery oxygen uptake (oxygen debt) was then 0 55 (0 54—0 58) which is higher than the value observed during continuous exercise (Asmussen 1946 Agnevik *et al* 1969) The explanation for this difference is most likely that in the present study relatively more energy was derived from phosphagen stores as they were replenished during rest periods

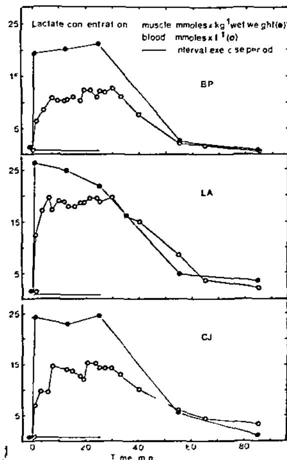


Fig 5 Lactate concentration in muscle tissue (same samples as in Fig 3 and 4) and blood for three subjects before, during and after intermittent exercise For comparison note that about 10—15 % of blood is made up of solids while the corresponding value for muscle tissue is 20—25 %

Heart rates observed during exercise were between 170 and 195 beats/min, i e close to their maximal values ($\approx$ 196 beats/min) The subjects might have reached their maximal oxygen uptake during the last part of the third, fourth and fifth bursts of activity to judge from the heart rate response

Discussion

The main purpose of the present investigation was to evaluate in what way the variables studied were related to the subjects perception of exhaustion The immediate sources of energy for muscle contractions are ATP and CP stores Complete depletion of these stores in the muscle would limit the ability of the muscle to contract No value under 1 5—2 0 mmoles$\times$kg^{-1} wet muscle has ever been observed for ATP in connection with maximal exercise in man (Hultman, Bergstrom and Mc Lennan Anderson 1967 Karlsson and Saltin 1970, Karlsson Diamant and Saltin 1970)

It might be argued that since biopsies are taken 1—3 sec after the last muscle contraction true exercise values are not obtained with the present method There is, however, a tendency for phosphagens to decline with each work period at the same time as there is an increase in the accumulated oxygen deficit The subjects were as exhausted after the first and third exercise burst as after the last, phosphagen concentration having fallen progressively thus suggesting that exhaustion cannot be attributed to a critically low phosphagen concentration in muscle

ADP concentrations were found to be reduced during interval period but again approached rest values during the recovery period Similar results were presented in another paper (Karlsson Diamant and Saltin 1970) and by other laboratories studying human skeletal muscle metabolism (Bergstrom *et al* 1971) ADP concentration is generally defined by its stoichometrical relationship to the ATP and the AMP concentration and equal amounts of ATP and AMP may be formed from ADP in the adenylate kinase reaction Thus ADP is a substrate in several reactions involving the resynthesis of ATP, and for this reason a lowering of ADP concentrations might take place during intensive muscular activity No stoichometrical agreement between ATP, ADP, and AMP has been found Moreover it has not been possible to demonstrate any increase in AMP On the other hand ATP lost to capillary blood (Forrester and Lind 1969) might provide a partial explanation of the discrepancy

The question of whether blood lactate or pH limit short term strenous physical exercise (Cerretelli 1967, Hermansen 1969) has been discussed Hermansen however, showed that there was a continuous increase in blood lactate and a concomitant decrease in blood pH in repeated bursts of maximal activity He therefore concluded that neither blood lactate nor blood pH were limiting factors During the first 15 min of the interval period in the present study there was a clearcut gradual increase in the blood lactate concentration With each exercise period a definite oxygen deficit on the order of 3 l of oxygen or more developed indicating the production of a large amount of lactate in each activity burst (Karlsson and Saltin 1970) In contrast to this lactate did not increase significantly after the first activity burst This might be explained in the following manner

1 In the course of time some of the lactate is distributed to water compartments other than those in the working muscles as indicated by the increase in blood lactate concentration
2 Lactate is also taken up and oxidized in different tissues of the body including the liver inactive muscles and fibers in the exercising muscles (Jorfeldt 1970)

Results have been published showing that trained people are able to achieve higher blood lactate concentrations during maximal exercise (Hermansen 1969 Karlsson Diamant and Saltin 1970) than untrained people and that training will increase the maximal lactate concentration in muscle (Saltin and Karlsson 1971) and in blood (Hermansen 1969) Moreover results are available (Kubler *et al* 196?) which indicate that the process for translocating lactate between muscle tissue and blood behaves only in part in accordance with the concept of passive diffusion The fact

that muscle lactate concentrations achieved their highest values early in the interval period and were well related to the subjects' perception of fatigue may favour the assumption that muscle lactate concentration in exhaustive exercise of brief duration is closely related, in any case, to the limiting factor The results from a previous study (Karlsson and Saltin 1970) with continuous, brief exhaustive exercise are in accordance with this assumption

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From the Department of Physiology, Gymnastik och idrottshögskolan, Stockholm, Sweden

Lactate in Working Muscles after Prolonged Exercice

By

JAN KARLSSON

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Abstract

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Nearly normal muscle lactate concentrations were obtained with a submaximal work load preceded by prolonged heavy exercise for 2—4 and 5—7 hrs resulting in a marked decrease in muscle glycogen content The same was true if prolonged work for 2—4 hrs preceded a standardized supramaximal work test in contrast to prolonged work for 5—7 hrs In all these studies however the blood lactate concentration was reduced after prolonged work, thus confirming earlier findings (Åstrand *et al* 1963) This might indicate enhanced utilization of blood borne lactate induced by the preceding prolonged work

In 1936 Bang reported that blood lactate concentration decreased during the latter part of work when a relatively heavy load was continued for more than 20—30 min According to Bang, this decrease indicated an increased utilization of lactate as a substrate by other tissues and organs

Åstrand *et al* (1963) found very low blood lactate values in contestants at the end of ski races lasting for 1 hr or more The same study demonstrated that blood lactate concentration was lower in standardized submaximal and maximal bicycle exercise performed after the ski race as compared with a control study before Åstrand *et al* (1963) proposed a reduced ability to produce lactic acid in these circumstances, which probably was not due to a low muscle glycogen level In an effort to study these questions further, lactate concentration was determined after prolonged heavy exercise both in blood and in muscle tissue with standardized submaximal and maximal work ATP, CP, ADP, P_i and glycogen concentrations in addition to lactate were determined using the same muscle specimens

Subjects and Methods

A total of 13 healthy physical education students were studied in two separate sets of experiments Pertinent data on the subjects are given in Table I All subjects were trained and accustomed to endurance exercise Some of them were also regular participants in competitions but none of them was especially trained for sprinting exercise

Abbreviations

ADP = adenosine 5'-diphosphate
ATP = adenosine 5' triphosphate
CP = creatine phosphate
P_i = inorganic phosphate

TABLE I Mean values and range for anthropological and experimental data in Series I and Series II

Series	Age years	Height cm	Weight kg	Max $\dot{V}_{O_2}$ $l \times min^{-1}$
Series I	23	180	68	4 1
n = 5	(22—24)	(172—189)	(65—75)	(3 4—4 7)
Series II	25	184	75	4 6
n = 8	(23—26)	(174—192)	(69—83)	(4 0—5 3)

A Krogh bicycle ergometer was used for standardized submaximal and maximal work, and the number of revolutions was automatically counted to make it possible to calculate the mechanical work accurately and to evaluate the oxygen deficit Submaximal and maximal work loads corresponding to about 75 per cent and 130—150 per cent respectively of the individuals maximal oxygen uptake were used (Table I) Oxygen uptake was determined with the Douglas bag method and the gas samples were analysed according to the Haldane technique Finger tip blood was obtained from a prewarmed hand for lactate determination which was made according to an enzymatic method introduced by S [illegible] obtained from the lateral portion of the quadriceps [illegible] biopsy technique (Bergstrom 1962) Biopsy specime[illegible] 3—5 sec after termination of exercise and stored in [illegible]

[illegible] tistical methods were [illegible] and the significance [illegible] intra individual dif[illegible]ferences

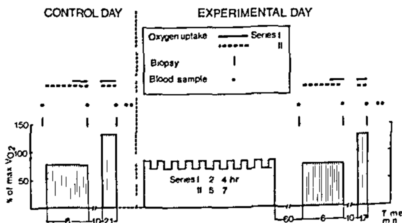


Fig 1 The experimental design Control day the subjects reported to the laboratory early in the morning and rested for 45—60 min in supine position whereupon rest samples were obtained The submaximal work load corresponding to 75 per cent of max $\dot{V}_{O_2}$ lasted for 6 min One biopsy sample and one blood sample were taken immediately after the completion of work After 10 min of rest sitting on the bicycle ergometer subjects performed supramaximal work corresponding to approximately 140 per cent of max $\dot{V}_{O_2}$ this was followed by the third biopsy and repeated blood [illegible] for 60

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Submaximal work		Maximal work		
Work load kpm × min⁻¹	Work time*	Work load kpm × min⁻¹	Work time*	[illegible]
1350 (1200—1500)	60 —	2500 (2400—2750)	1.53 (1.41—1.[illegible])	1.2 [illegible]
1570 (1350—1800)	60 —	2680 (2400—2850)	2.3 (2.16—3.[illegible])	[illegible]

* in min

Procedure

[illegible]

comitantly taken for blood lactate determination [illegible] After 10 [illegible]

[illegible]

Results

In Table II and III the mean values for the stud[illegible] the submaximal and the maximal work loads were perf[illegible] exercise, no statistically significant difference in mu[illegible] AT [illegible] compared to [illegible]

kg⁻¹ ($p>0.05$), for the muscle lactate in Series I, b[illegible] Series II as in the control study (≈ 11 mmoles × kg⁻¹)

With the maximal work load muscle tissue lactate [illegible] Series I after the prolonged exercise period which [illegible] in the control study (≈ 23 mmoles × kg⁻¹), but values [illegible] lower ($p<0.001$) were found in Series II

Although muscle lactate concentration tended to [illegible] load, blood lactate concentration tended to be [illegible] l⁻¹ in the control study to 4.2 mmoles × l⁻¹ in Series I [illegible] l⁻¹ in Series II $p<0.01$) With the maximal load a [illegible] present and amounted to 30 and 40 per cent [illegible] studies

TABLE II Mean values ± S E and S D for ATP, CP, ADP, and P_i at rest and after two standardized work loads for the control study (Series I + Series II), and for Series I and Series II respectively after prolonged heavy exercise The level of significance denotes the difference based on intraindividual comparisons between the control experiments and those performed after the preceding endurance exercise

Metabolite		Values obtained in the control study n = 13 (Karlsson *et al* 1970)	Values obtained after 2—4 hrs' exercise n = 5		Values obtained after 5—7 hrs exercise n = 8	
Adenosinetriphosphate (ATP) mmoles × kg⁻¹ wet muscle	Rest	4 7±0 2 0 6	4 8±0 2 0 5	p > 0 5	4 1±0 2 0 4	p > 0 5
	75 %	3 9±0 2 0 6	3 9±0 3 0 6	p > 0 5	4 0±0 2 0 4	p > 0 5
	Max	2 8±0 1 0 3	2 6±0 3 0 7	p > 0 5	2 2±0 4 1 1	p > 0 5
Creatinephosphate (CP) mmoles × kg⁻¹ wet muscle	Rest	14 5±0 4 1 5	13 0±1 2 2 7	p > 0 5	13 8±1 1 3 2	p > 0 5
	75 %	6 4±0 4 1 4	5 9±0 4 0 8	p > 0 5	6 2±0 9 2 6	p > 0 5
	Max	2 9±0 2 0 6	3 3±0 4 0 9	p > 0 5	4 0±0 9 2 7	p > 0 5
Adenosinediphosphate (ADP) mmoles × kg⁻¹ wet muscle	Rest	1 6±0 1 0 3	1 4±0 1 0.2	p > 0 5	1 8±0 1 0 2	p > 0 5
	75 %	1 3±0 1 0 3	0 9±0 1 0 2	p > 0 5	1 5±0 1 0 2	p > 0 5
	Max	1 0±0 1 0 3	0 6±0 1 0 2	p > 0 5	0 9±0 2 0 5	p > 0 5
Inorganic phosphate (P_i) mmoles × kg⁻¹ wet muscle	Rest	10 2±0 5 1 7	10 6±0 5 1 2	p > 0 5	10 8±0 4 1 3	p > 0 5
	75 %	15 2±0 8 2 9	17 4±1 1 2 4	p > 0 5	14 5±0 8 2 2	p > 0 5
	Max	10 1±1 0 3 7	14 4±0 1 1 7	p > 0 5	9 8±0 9 2 5	p > 0 5

In the control study the glycogen concentration averaged 74 mmoles glucose units × kg⁻¹ wet muscle and was depleted to 62 and 42 mmoles × kg⁻¹ at the submaximal and the maximal work load, respectively In Series I the glycogen content was as high as 58 mmoles × kg⁻¹ after the prolonged exercise A decrease to 38 and 25 mmoles × kg⁻¹ wet muscle respectively was observed when the standardized work was performed. The corresponding figures in Series II were 65, 44 and 22 mmoles × kg⁻¹ wet muscle respectively From these data it is clear the glycogen depletion was similar in all three experiment series

TABLE III Mean values ± S E and S D for muscle lactate blood lactate and oxygen deficit for Series I and Series II at rest and after the two standardized work loads in the control study and after prolonged heavy exercise respectively The level of significance denotes as in Table II the difference based on intraindividual comparisons between the control study and the studies performed after prolonged exercise respectively

		Series I n 5		Series II n = 8	
		before	after	before	after
Muscle lactate mmoles × kg⁻¹ wet muscle	Rest	1 5±0 8 1 9	1 6±0 2 0 5, p > 0 05	1 4±0 2 0 5	1 7±0 3 0 8 p > 0 05
	75 % of max $\dot{V}o_2$	11 4±2 0 4 4	13 5±2 5 5 5, p > 0 05	10 6±1 4 4 0	10 7±1 4 4 0 p > 0 05
	Max	23 9±3 5 7 8	22 0±1 5 3 4, p > 0 05	22 0±1 7 4 9	13 3±1 3 3 7, p < 0 01
Blood lactate mmoles × l⁻¹	Rest	1 0±0 3 0 6	1 3±0 1 0 2 p > 0 05	1 4±0 2 0 7	1 9±0 1 0 1, p > 0 05
	75 % of max $\dot{V}o_2$	5 7±0 2 0 5	4 2±0 8 1 8 p > 0 05	5 1±0 4 1 2	3 9±0 5 1 4, p < 0 01
	Max	14 2±1 1 2 4	10 1±0 9 2 1, p < 0 01	14 9±0 7 1 9	9 4±1 2 3 4 p < 0 001
O_2 Deficit l	75 % of max $\dot{V}o_2$	—	—	3 3	4 7, p < 0 01
	Max	3 9	3 6	5 8	4 7 p < 0 01

Discussion

The present results indicated that a preceding prolonged period of heavy exercise did not influence ATP, CP, ADP and P_i concentrations at the standardized work loads *i e* alactacid anaerobic energy output was the same as in the control study It has been demonstrated in animals and in man that prolonged exercise can induce morphological changes of the mitochondria (Gollnick *et al* 1969 Gollnick and King 1969) Thus, swelling as well as complete destruction of the cristae have beeen shown to occur In vitro experiments on mitochondria have shown that morphological structural changes will result in an impairment of the efficiency of ATP oxidative resynthesis *i e* a decreased P/O ratio (Weinback Garbus and Sheffield 1967) Our data showing no change in ATP and CP concentrations and normal mechanical efficiency during exercise may then indicate that no changes of the kind reported by Gollnick and King occurred in our subjects This is in line with the findings (Gollnick, Ianuzzo and King 1971) that mitochondrial changes mainly occur in untrained men and animals our subjects were used to regular exercise

After prolonged heavy exercise in the present experiments blood lactate concentration was reduced in submaximal and maximal exercise However muscle lactate

concentration was the same as observed in the control study with the exception of the maximal exercise in Series II In fact 4 out of 5 subjects had an elevated muscle lactate concentration with the submaximal exercise in Series I indicating increased anaerobic glycolysis After endurance work there was an increased oxygen deficit at the submaximal work load in Series II due to slower acceleration of oxygen uptake at onset of work

As there is a good correlation between oxygen deficit and muscle lactate formation with heavy work (Karlsson and Saltin 1970) higher muscle lactate concentrations might also be expected in Series II in the submaximal work However, this was not the case This contradictionary observation may be explained by the decreased blood lactate concentration indicating enhanced utilization of lactate induced by the preceding prolonged heavy exercise This must facilitate the flux of lactate from the working muscle and diminish the degree of accumulation in the muscle More over 4 of 5 subjects had a lower blood lactate concentration despite the tendency for muscle lactate concentration to increase at the submaximal work load in Series I It seems likely that an underestimation of the actual amount of lactate produced might be deduced from the blood lactate determinations obtained with work per formed after a preceding period of prolonged exercise

The picture obtained with the maximal load is not so clear In Series I no signifi cant reduction in muscle lactate concentration was found as might be expected from the unchanged oxygen deficit but a substantial decrease in muscle lactate concentra tion was observed in Series II In Series II however oxygen deficit during maximal exercise was less than the control data According to Karlsson and Saltin (1970) this would result in a maximum lower lactate accumulation of about 5 mmoles $\times$ kg^{-1} wet muscle

Other factors must be introduced to explain the low muscle lactate concentration in Series I and II The lower blood lactate concentration may be one factor as pointed out above but since no marked decrease in muscle lactate concentration was seen in Series I despite the decreased blood lactate concentration other factors may also be of significance One such factor might be the extremely brief work time (1 2 min which would limit the influence of a more efficient lactate flux induced by the endurance work observed in Series I at the maximal work load

It might be thought that completely depleted or nearly depleted glycogen stores would limit lactate formation and thus explain the impaired anaerobic work observed with the maximal load in Series II As demonstrated by Saltin and Hermansen (1967) a decreased blood lactate concentration with brief maximal bicycle work indicating impaired anaerobic glycolysis will not be found until nonmeasurable amounts of glycogen are present in the examined muscle

The glycogen concentration in the muscle examined prior to the tests was lowered in both the series as a consequence of the preceding prolonged heavy exercise However in spite of the rather long endurance work in both Series I and II rather large amounts of glycogen were left in the examined muscle Consequently glycogen was present in amounts great enough to secure at least normal lactate formation with the

standardized submaximal and maximal work loads and no glycogen content less than 11 mmoles of glucose units×kg⁻¹ wet muscle was obtained in any subject after maximal work

It might be questioned as to whether or not a muscle group with such high amounts of glycogen remaining in the muscle tissue can validly be used for examination of metabolic changes induced by preceding prolonged exercise period In Series I the endurance work consisted of running and of cross country skiing in Series II Caloric output during the work was estimated at approximately 2800 and 5800 kcal, respectively It is very likely that the subjects started the endurance work with elevated glycogen values which they had attained by following a special diet (Saltin and Hermansen 1967) the day preceding the experiment Thus depletion due to endurance work would be higher than just the difference between a normal glycogen concentration (*i e* the basal level in the control study) and the muscle glycogen level observed after the prolonged exercise in Series I and Series II In addition the type of work is another factor explaining the large amount of glycogen left in the muscle examined In activities such as skiing and running an incomplete depletion of glycogen in the lateral part of the quadriceps muscle has been demonstrated (Karlsson and Saltin 1971 and unpublished observations), this is in contrast to bicycle work (Hermansen Hultman and Saltin 1967)

As mentioned above the present results on the effect of prolonged exercise on blood lactate concentration after submaximal and maximal exercise are generally comparable to earlier studies (Bang 1936 Åstrand *et al* 1963 Saltin 1964) in this field On the other hand a much more pronounced reduction in the blood lactate concentration especially after the maximal exercise was observed in previous investigations An uptake and oxidization of lactate in skeletal muscles have been demonstrated (Jorfeldt 1970 Stainsby and Welch 1966) in both human and animal specimens Studies in progress also show that a period of exercise enhances the turn over rate of infused lactate as a result of a more pronounced uptake in skeletal muscles Moreover it has been demonstrated that a previous period of prolonged exercise increases LDH activity (Karlson Diamant and Saltin 1968) This is most probably due to a predominant increase in the activity of the M forms of the enzyme but it cannot be excluded that the same was also true for the H forms which favour the oxidation of lactate These findings indicate that even rather brief periods of exercise can markedly affect muscle metabolism The reason for the lower blood lactate concentration observed earlier as compared with present data is then most likely that the time which passed until exhaustion at the maximal work load was 4—8 min in Åstrand *et al* s and Saltin s studies and only around 1 5 min in the present one It may then be concluded that after prolonged exercise marked changes in the ability to produce lactate do not occur although maximal muscle lactate concentrations were not observed after extremely prolonged exercise The low blood lactate concentration observed is the result of a faster turn-over of lactate in the body However an explanation is still required as to why subjects are more easily exhausted after a prolonged period of exercise

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From the Department of Physiology, Karolinska Institutet, Stockholm Sweden

The Effect of Deuterium on the Release and Uptake of Noradrenaline in Isolated Nerve Granules

By

U S VON EULER and F LISHAJKO

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Abstract

EULER, U S v and F LISHAJKO *The effect of deuterium on the release and uptake of noradrenaline in isolated nerve granules* Acta physiol scand 1971 *82* 131—134

The release of noradrenaline (NA) from isolated bovine splenic granules in phosphate buffer is decreased by 10—25 per cent by addition of 50—90 % D_2O to the medium The accompanying uptake of ^{14}C(-)NA is unchanged in 50—70 % D_2O and sligthtly decreased in 95 % D_2O After previous partial NA depletion of the granules the net uptake of NA in the presence of ATP Mg^{2+} is lowered by D_2O in concentrations above 25 % in the medium and has fallen to 63 per cent of normal with 87 5 % D_2O

Incubation of isolated nerve granules from bovine splenic nerves in isotonic phosphate buffer pH 7—7 5 causes a release of noradrenaline (NA) at a rate of about 2 per cent of the bound transmitter per minute at 20° and about 15 per cent per minute at 37° The release is accompanied by reuptake in the presence of NA in the medium from about 10^{-7} M (Euler and Lishajko 1967), with a K_m of approximately $1\ 5\times10^{-6}$ M (Euler 1970) After previous partial depletion an ATP facilitated net uptake with a closely similar K_m value is observed on continued incubation which may restore the NA content to preincubation values or higher in 20—30 min at 20° (Euler and Lishajko 1969)

In the experiments to be reported here the effect of substitution of D_2O for H_2O in the medium has been studied

Material and Methods

Bovine splenic nerves were obtained from the slaughter house and transported on ice to the laboratory After homogenization of the desheathed nerves in an Ultra Turrax apparatus (Janke and Kunkel Freiburg) at moderate speed for 15—30 sec in 10 volumes icecold 0 13 M potassium phosphate at pH 7 5 the suspension was centrifuged at +2 at $9\ 000\times g$ for 10 min for removal of coarse material and larger particles Aliquots of the supernatant, containing

TABLE I Release experiments Remaining NA in nerve granules after incubation for 60 min at 20° in 0 13 M phosphate buffer at pH 7 5 with varying concentrations of D_2O and incorporated radioactive NA, in per cent of control Mean and SEM

Per cent D_2O	0	25	50	75	95
Remaining NA in granules after incubation in per cent of control (n = 4)	100	102±2 9	115±5 0	125±8 1	111±7 7
Incorporated ^{14}C-NA in per cent of control (n 3)	100	105	104±2 9	104±2 8	89±2 5

[1] 1 expt

most of the granule bound NA were further centrifuged for 30 min at 50 000×g The granule pellets were resuspended in phosphate buffer with varying concentrations of D_2O to a final volume of 2 5 ml and the suspension incubated for 1 hr at 20° in the release experiments ^{14}C-(−) NA (Radiochem Centre Amersham 57 mCi/mmole) was added to approximately 2×10^{-6} M before incubation

granules were further incubated for 30 min at 20° in phosphate buffer containing varying proportions of D_2O with addition of non labelled (−) NA to 10^{-5} M ATP and $MgCl_2$ each 3 mM and ^{14}C (−) NA 2×10^{-6} M in a total volume of 8 ml

Phosphate buffer was made with deuterium oxide (Norsk Hydro-Elektrisk Kvaelstof A/S Oslo) and used as incubation medium either undiluted or together with ordinary phosphate buffer giving final concentrations of 25 50 75 or 87 5 % D_2O in the net uptake experiments and 25—95 % D_2O in the release experiments

After incubation the suspension was centrifuged in a refrigerated centrifuge for 30 min at 50 000×g The granule pellet was washed and resuspended in fresh ordinary phosphate buffer and recentrifuged After extraction with 0 4 M perchloric acid NA in the extract was determined fluorimetrically

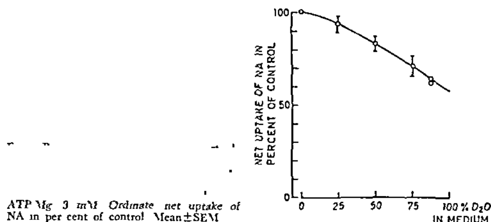
Radioactivity was determined in the supernatant and in the extract of the pellet using a Packard spectrometer and scintillation fluid as described previously (Euler and Lishajko 1967)

Results

Release experiments

In the release experiments the NA amounts remaining after incubation for 1 hr at 20 in the presence in D O concentrations 25 50 75 and 95 per cent were determined (Table I

From Table I it can be seen that whereas a concentration of 25 % D O in the medium has no apparent effect on the release rate 50, 75 and 95 % D O caused a moderate decrease in the net release The relative incorporation of radioactive NA was not significantly altered at 25—75 % D O indicating that the reuptake process was largely unimpaired At 95 % D O the incorporation of labelled (−) NA was significantly decreased however



ATP Mg 3 mM Ordinate net uptake of NA in per cent of control Mean ± SEM

Net uptake experiments

In these experiments the ATP-Mg facilitated net uptake of NA was determined in the presence of varying concentrations of D_2O in the medium The effect of D_2O was dependent on the concentration and increased with increasing proportions of D_2O As *in the release experiments* D_2O *in a concentration of* 25 % *had no significant effect*, whereas a concentration of 87 5 % D_2O, which was the highest studied, decreased the net uptake to about 60—65 % of the control uptake (Fig 1)

The incorporation of labelled (—) NA followed fairly closely the net uptake of NA as seen in Table II

Comment

The mechanism of amine release or uptake in isolated nerve granules is not known in details The facilitating effect of ATP (and of ADP UTP ITP and CTP) in the presence of Mg^{2+} on reuptake as well as net uptake of NA is however well established (Euler and Lishajko 1969)

TABLE II Net uptake of total and of labelled ( ) NA in partially depleted splenic nerve granules in a medium containing varying concentrations of D_2O Mean and SEM ATP Mg 3mM and NA 10^{-4} M n = number of expts

Conc D_2O in medium	n	Uptake in per cent of control	
		Total (NA	Labelled ( ) NA
0 %		100	100
25 %	(6	94 4 3	94 ± 2 8
50 %	(6	83 4 2	81 − 2 6
75 %	6	71 ± 5 5	72 ± 2 8
87 5 %	(?	63 (62 64)	68 (66 69)

Uncouplers and inhibitors of oxidative phosphorylation and the respiratory chain have been found to greatly influence the spontaneous release as well as reuptake and net uptake in isolated nerve granules These findings suggested that both these processes are metabolically dependent Various compounds known to affect membrane structures also influence the NA release as well as the NA uptake It therefore appeared possible that the uptake system of the granules might be affected by substituting D_2O in the medium for H_2O

Numerous reports have described inhibitory effects of D_2O on ezyme systems *in vivo or in vitro Depending on the conditions these effects may be either solvent* effects or associated with deuteration of the substrate (cf Belleau *et al* 1960, Katz 1960, Kritchevsky 1960, Thomson 1963)

Appreciable inhibition of enzyme activity has been noted at high D_2O concentrations, thus Svensmark (1961) observed 45 per cent inhibition of the activity of muscle ATP ase in 85—90 % D_2O At concentrations of the order of 10—30 % D_2O the inhibitory effects on enzymatic reactions have generally been moderate or *small*

In the system studied in this report it seems probable that D_2O acts on a granule-bound multi enzyme system, in wich both isotope and solvent effects may contribute to the effect The demonstration of about 35—40 per cent decrease of the ATP Mg facilitated net uptake of NA in isolated nerve granules with 87 5 % D_2O further supports the concept that these processes are associated with metabolic enzyme reactions

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From the Department of Medical Chemistry, University of Helsinki, Finland

Characterization of Neuronal and Glial Fractions Separated in Sucrose and Ficoll Media

By

K Hemminki and E Holmila

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Abstract

Hemminki K. and E Holmila *Characterization of neuronal and glial fractions separated in sucrose and Ficoll media* Acta physiol scand 1971 *82* 135—142

Rat brain cerebral cortices were dispersed by a collagenase hyaluronidase treatment and the

were clearly more active in neuronal fractions In Krebs-Ringer phosphate buffer containing 10 mM glucose, neurons respired 946 and 875 and glia 706 and 396 nmoles oxygen/mg prot/hr after sucrose and Ficoll separation respectively Incorporation of radioactive leucine into protein was 5370 and 4980 dpm/mg prot for neurons and 3110 and 4480 dpm/mg prot for glia On the other hand no clear difference was found in TCA soluble radioactivities between neuronal and glial fractions

Bulk preparation of neurons and glia is now attempted in many laboratories to elucidate the metabolic contributions of the two cell types to the function of the brain At present such an approach poses three kinds of restrictions condition of the cells amount obtained and purity of the fractions Recently some enzymatic and sieving methods for preparing brain cell suspensions were studied in our laboratory (Hemminki *et al* 1970) and a new method based on collagenase hyaluronidase digestion was presented (Hemminki 1970) This was found satisfactory both as to the condition of the cells and the yield Adopting the collagenase hyaluronidase method for dispersion the separation of neurons and glia was studied A crude fractionation of neurons and glia was achieved by a rapid sedimentation centrifugation Combining this with a sucrose density gradient an 80—85 % purity of the neuronal and glial fractions was obtained The sucrose separation was compared to a modification of a Ficoll gradient Rose 1967 Blomstrand and Hamberger 1969)

Cells from the two separation techniques were subjected to metabolic studies in vitro, and a distinct metabolic differentiation between neuronal and glial fractions was established

Materials and Methods

Animals 5 Albino Wistar strain rats around one month old, were used in each experiment

Materials Collagenase 194 U/mg (crude fraction), was obtained from Worthington and hyaluronidase type I from Sigma L leucine 4 5 ^{3}H (sp a 55 2 C/mmole) was from NEN Ficoll molecular weight 400 000 batch 4540, was from Pharmacia

Glass-distilled water was used throughout

Solutions Sucrose and Ficoll solutions used for gradients were made in 10 mM phosphate buffer pH 7 4 also containing 10 mM glucose For other purposes Krebs Ringer phosphate

ed previously (Hemminki with the enzyme and the collagenase concentration was increased to 0 05 %

After the enzyme incubation was completed cells were sedimented at 300×*g* and 4° C for 5 min and the supernatant was decanted

Sucrose separation 9 ml

10 ml were layered onto 10 ml of 1 1 M sucrose on top of 5 ml of 1 4 M sucrose tube (tube 1) The third 10 ml were layered onto 5 ml of 1 1 M sucrose on top of 1 5 M and 2 M sucrose (tube 2) The remaining 5 ml were mixed with 10 ml of 1 5 M sucrose and layed onto 1 5 M sucrose on top of 2 M sucrose (tube 3) The three tubes were spun at 6000 rpm (5000×*g*) in a Spinco SW 25 rotor for 20 min The interfaces between 1 1 M—1 4 M (A) and 1 1 M—1 5 M sucrose (B) were collected by a Pasteur pipette and represented the glial fraction The interfaces between 1 5 M—2 M sucrose (C and D) represented the neuronal fraction Both fractions were slowly diluted 5 times by Krebs Ringer phosphate containing 0 32 M sucrose Cells were collected by centrifugation at 800×*g* for 10 min The resultant pellets were washed twice with Krebs Ringer phosphate and sedimented at 500×*g*

Ficoll separation 30 ml of 25 % Ficoll were added to the 300×*g* pellet and the pellet was resuspended by sucking it back and forth in a pipette 10 ml aliquots of the suspension were divided between three 35 ml tubes All tubes contained the same layers 5 ml of 2 M sucrose at the bottom 5 ml of 30 % Ficoll 10 ml of the sample 5 ml of 20 % Ficoll and 5 ml of 10 % Ficoll at the top The tubes were spun in a Spinco SW 25 rotor at 25 000 rpm (63 000×*g* for 90 min The interfacial layer between 10 % and 20 % Ficoll was collected as the glial fraction and the layer between 30 % Ficoll and 2 M sucrose as the neuronal fraction The fractions were slowly diluted 3 fold with 5 % Ficoll and centrifuged at 1000×*g* for 10 min The resultant pellets were washed twice with Krebs-Ringer phosphate buffer and sedimented at 500×*g*

Microscopy Methyle blue was used for staining and samples were examined and photographed by a light microscope Cell counts were taken on a heamocytometer 40 fields were counted from the neuronal and glial fractions obtained by 4 seperate centrifugations Particles observed were classified in following categories neurons glia red cells capillary cells nuclei and fibre material A percentage of each type was then calculated from a total of 800 particles Unclassified objects (less than 3 % of the total) and small depris were not counted In this context the purity is defined as the percentage of the neuronal or glial cells in the neuronal or

Fig 1 Scheme for filling tubes in sucrose centrifugation

glial fractions, respectively Average cellular protein content was calculated by subtracting the estimated protein of the contaminating material from the total protein of the fraction divided by cell number

Respiratory experiments Experiments were performed in a Warburg respirometer, each flask containing 2 ml of cell suspension in Krebs-Ringer phosphate buffer, pH 7 4, containing 10 mM glucose Flasks were gassed with O_2 and shaken at a rate of 100 per min Incubation was continued for 90 min

Measurement of radioactivities Flasks containing 2 ml of cell suspension in Krebs Ringer phosphate buffer, pH 7 4, 10 mM glucose and 1 mM leucine were shaken in a water bath (37° C) gassing with O_2 After a 10 min preincubation 5 μC of ^{3}H leucine (s p 55 2 C/mmole) wer [illegible] ued for 60 min Experiments w [illegible] ing the contents into 50 ml of [illegible] mM glucose in which K⁺ had [illegible] for 3 min The supernatants were carefully decanted and the walls of the tubes were wiped dry with paper The pellets were removed with 4 ml of cold 5 % TCA into a Potter Elvehjem homogenizer The TCA homogenates were centrifuged and the supernatants kept for measurement of TCA soluble radioactivity and potassium The pellets were washed as described previously (Hemminki 1970) and the radioactivity and protein content of the TCA precipitable material were determined Protein was measured according to Lowry (Lowry *et al* 1951) Radioactivities were recorded in a Packard two-channel liquid scintillation spectrometer

Measurement of potassium The potassium content of the TCA solutions was determined by the Baird Atomic flame photometer Results were expressed as μmoles K⁺/mg protein

Results

Recovery of cells and protein

The collagenase hyaluronidase method for preparing brain cell suspension produced some 30×10^6 neurons and 24×10^6 glia per g of wet tissue (Hemminki 1970) When a mixed cell suspension was separated in a sucrose system some 6×10 neurons and 17×10^6 glia were recovered per g wet weight (Table I) In Ficoll the recovery was some 19×10^6 neurons and glia 5 rat brain cortices, cleaned of white matter, contained around 150 mg of protein In sucrose 4,5 mg and in Ficoll 5 5 mg of protein was obtained in the two fractions These present a yield of 3—3 5 %

Protein content of a cell

Average protein content of a cell deviated somewhat depending on the separation method used (Table I) After sucrose both cell types seemed to contain 70 pg prot/cell, after Ficoll the protein content was 55—50 pg prot/cell

TABLE I Number of recovered neurons and glia separated from 5 rat cortices (c 2 g wet tissue) and calculated cellular protein content of the obtained cells Separation performed in sucrose and Ficoll systems 4 different gradients were analysed Means ± S E M

	Recovered cells millions		Protein per cell pg)	
	sucrose	Ficoll	sucrose	Ficoll
Neurons	12 ± ?	37 5	70 15	55 ± 5
Glia	34 5	3 6	0 ± 10	50 ± 10

TABLE II Distribution of cells in sucrose sedimentation run Aliquots from 4 different centrifugations were analysed Means ± S E M

Aliquot	Number of cells in millions	
	Neurons	Glia
7 ml (top)	2±1	4±1
10 ml	9±3	14±3
10 ml	13±3	19±4
5 ml (bottom)	31±6	11±3

TABLE III Classification of particles by light microscopy in neuronal and glial fractions separated in sucrose and Ficoll systems (%) 4 different gradients were analysed Means ± S E M

	Sucrose		Ficoll	
	neuronal	glial	neuronal	glial
Neurons	82±5	10±2	61±7	4±1
Glia	9±2	84±5	24±3	69±7
Red cells	—	—	6±1	
Capillary cells	5±2	—	5±1	6±1
Nuclei	4±1	—	4±1	—
Fibre material	—	6±2	—	21±4

Distribution of particles after sucrose sedimentation and in final fractions

To investigate the separation properties of the sucrose sedimentation run particle distribution in 4 aliquots was counted on a haemocytometer (Table II) The 5 ml aliquot at the bottom contained over half of the neurons Two 10 ml aliquots in the middle contained most of the glia The uppermost 7 ml contained fibre material, red cells and capillary cells were found in each of the three aliquots at the bottom Particle counts were taken on final cell fractions (Table III) Fractions separated by sucrose were observed to be 80—85 % pure and those separated by Ficoll 60—70 % pure

Microscopic observations of the fractions

Fig 2—7 show photographs of the isolated fractions Neurons appear much alike independent of the separation technique applied They have lost most of their processes during the tissue disruption and except for some chromatolysis no observable changes took place during the density gradient centrifugation It is possible, however, that the high osmolarities of the sucrose solutions are disastrous to some neurons because they are so much reduced in number Glial cells have retained their integrity to a larger extent and no changes can be observed in them by light microscopy



Fig 2 Neuronal fraction from sucrose gradient ×400 Methylene blue staining
Fig 3 Neuronal fraction from Ficoll gradient ×400 Methylene blue staining
Fig 4 Glial fraction from sucrose gradient ×400 Methylene blue staining
Fig 5 Glial fraction from Ficoll gradient ×400 Methylene blue staining
Fig 6 Two neurons from sucrose gradient ×1000 Methylene blue staining
Fig 7 A glial cell from sucrose gradient ×1000 Metylene blue staining
All preparations are unfixed and embedded in Canada balsam

Potassium

Potassium in the cellular pellets was determined to estimate the ability of cells to retain a potassium gradient (Table IV) Sucrose separation produced cells containing 0 32—0 33 μmoles K^+/mg prot Neurons separated in Ficoll retained K^+ 0 29 μmoles/mg prot but glia showed a higher K^+ content 0 45 μmoles/mg prot.

TABLE IV Recovery of potassium respiration TCA precipitable and TCA soluble radioactivities of neurons and glia separated in sucrose and Ficoll systems ± S.E.M. (n 3—4)

	Potassium recovered μmoles/mg prot		Respiration nmoles O_2/hr/mg prot	
	sucrose	Ficoll	sucrose	Ficoll
neurons	0.33 ± 0.04	0.29 ± 0.03	946 ± 108	875 ± 70
glia	0.32 ± 0.02	0.45 ± 0.05	706 ± 49	396 ± 35
	TCA precipitable radioactivity dpm/mg prot		TCA soluble radioactivity dpm/mg prot × 10^4	
	sucrose	Ficoll	sucrose	Ficoll
neurons	5370 ± 340	4980 ± 410	6.82 ± 0.42	2.24 ± 0.17
glia	3110 ± 230	4480 ± 320	6.92 ± 0.28	4.29 ± 0.41

Metabolic experiments

Respiration was more active in fractions prepared in sucrose (Table IV). A clear difference was recorded between neuronal and glial fractions independent of the separation technique used. In fractions separated by sucrose uptake of O was 34 % higher in neurons than in glia. Ficoll separation resulted in 120 % higher values for neurons than for glia. O uptake by neurons was 946 and 875 nmol/hr/mg prot in sucrose and Ficoll fractions respectively.

Incorporation of radioactive leucine into protein did not differ as much between the separation techniques. After sucrose separation neurons incorporated 70 % more radioactivity than glia. After Ficoll separation the difference amounted only to 11 % (Table IV).

High activities were recorded in the TCA soluble pool. Fractions separated in sucrose accumulated radioactive leucine around 68 000 dpm/mg prot. After Ficoll separation activities recorded were 1/3 for neurons and 2/3 for glia of the corresponding values of sucrose separation (Table IV).

Discussion

Several separation techniques have been described for the isolation of neurons and glia in large quantities. In some Ficoll is used as a separation medium (Bocci 1966, Rose 1967, Blomstrand and Hamberger 1969), in some others (Satake and Abe 1966, Norton and Poduslo 1970) sucrose is used. Quite controversial appraisals of the separation properties of Ficoll have been published. A marked heterogeneity of the fractions was reported by Cremer *et al.* (1968) whereas relatively high purity was stated by Blomstrand and Hamberger combining a low speed centrifugation with a Ficoll density gradient. In our experiments in spite of the 300×g centrifugation in the beginning only some 60—70 % purity was obtained. This is in agreement with recent reports by Rose and Sinha (1969, 1970). For a sucrose gradient 90 % purity of the neuronal and 70 % purity of the glial fraction

was reported by Norton *et al* In our hands the technique described by Norton *et al* was not as encouraging and gradients were easily obstructed by overloading with material To avoid trapping effect and cross-contamination a rapid separation method of neurons and glia was worked out based on sedimentation properties Combination of the two separation factors, sedimentation and density, resulted in a marked decrease of cross contamination A sedimentation analysis was also made in Ficoll (5—10 % concn were tested) but it was found ineffective in separating neurons and glia

Microscopic observations did not reveal any clear changes in cells separated in sucrose or Ficoll media, neither did the protein content per cell differ very much The average cell protein content was 50—70 pg/cell These are within the range reported by Satake and Abe (1966) and Freysz *et al* (1968), but are much smaller than those quoted by Rose (1967) Proteolytic enzymes were used by us for tissue disruption and this may explain some of the discrepancy However, in a previous paper (Hemminki *et al* 1970), protein concentrations were correlated to DNA values and they were consistent with DNA protein values reported by Zamenhof *et al* (1964) The high cellular protein content quoted by Rose could partly be due to a large amount of free subcellular organelles distributed in the collected fractions

Metabolic differences between fractions separated in sucrose and Ficoll can probably be explained in two ways One consists of the specific effects of the separation media on cellular structures and metabolic functions, the other depends on the different separation properties of the media, and hence differences in actual cellular (and organelle) populations A marked distinction was observed between neuronal and glial activites Neurons were more active both in uptake of oxygen and incorporation of radioactive leucine into protein In earlier respiratory experiments with cell suspensions (Rose 1967) no clear difference was observed between neurons and glia By other techniques highly varying results have been obtained (Elliot and Heller 1957, Korey and Orchen 1956, Hamberger 1963) Blomstrand *et al* (1969) studying the incorporation of leucine into protein in vivo reported three times higher activites for the neuronal fraction than for the glial one, parallelling our findings *in vitro* The high TCA soluble redioactivities and cellular K centents indicate the existence of membrane functions For fractions isolated in sucrose the TCA soluble radioactivity and K-content correspond to around 60 % of the values measured with brain slices (Huttunen 1969)

In this study some basic metabolic properties of the neuronal and glial fractions were characterized The data collected show high functional activities of the preparations However light microscopy cannot answer the questions concerning the structural integrity of the cells or the purity of the fractions from free subcellular contamination Further investigations have been launched to answer these questions using electron microscopy

We wish to thank Professor J Jarnefelt for suggestions concerning the manuscript and S E Jansson M D for taking the photographs This study was supported by the Sigrid Juselius Foundation and the National Council for Medical Sciences

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Acta physiol scand 1971 82 143—144

From the Department of Surgery Aker Hospital Oslo and the Institute of Zoophysiology University of Oslo Norway

The Effect of Breathing on the Cardiovascular Adjustments Induced by Face Immersion in Man

By

ARNOLDUS SCHYTTE BLIX JOHN KROG AND HANS OLAV MYHRE

Andersen (1963) has reported that bilateral interruption of the trigeminal nerve abolishes submersion bradycardia typical of ducks A slowing of the heart upon face immersion of man has later been reported by several authors (Craig 1963, Elsner *et al* 1963 Brick 1966 Pauley 1969 and Song *et al* 1969) Arrest of breathing however is obviously a consequence of face immersion of normal subjects It would therefore be of interest to repeat these experiments in a person who has the ability to continue to breathe normally while his head is kept under water *i e* a tracheostomized man Studies of the heart rate related to face immersion in this person may furnish valuable information concerning the afferent pathways involved in the establishment of the cardio vascular adjustments to diving in man

Material

forehead to avoid disturbance from the muscle activity during immersion Heart rate was recorded by means of an electrocardiograph

Results

The results from face immersion and breath holding is shown in Fig 1 No bradycardia was observed from face immersion while breathing normally through the tracheostomy in our experimental subject Apneic face immersion however caused a moderate decrease in heart rate in the same person Considerable decrease in heart rate was found during breathholding and apneic face immersion in the control It was also observed that the decrease in heart rate of the control subject was dependent upon where in the respiratory cycle breath holding was initiated Diving following expiration caused less slowing of the heart than dividing with fully extended lungs

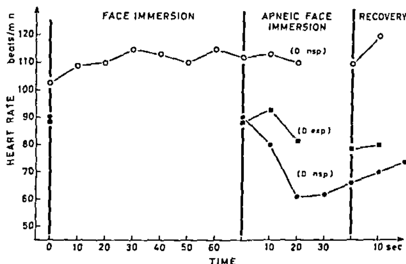


Fig 1 Heart rate during face immersion and breath holding in tracheostomized (0—0), and normal man, (●—●) prior to inspiration (■—■) after expiration

Conclusion

It is our opinion that the slowing of the human heart during face immersion is at least partly due to lack of movement of the thorax and that the bradycardia is vagally induced as a result of peripheral vasoconstriction secondary to the breath holding (Cohn *et al* 1968, Blix & Myhre)

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Acta physiol. scand. 1971. 82. 145—153

From the Department of Physiology, Veterinärhögskolan, Stockholm, Sweden

The Effect of Changes in the Blood Flow to the Tongue on the Chorda Tympani Response in Rat

By

GORAN HELLEKANT

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Abstract

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cussed here

Over the last decade it has been increasingly revealed that the mammalian taste bud is a very active tissue This is suggested by the observation that its rate of cell division is high (Beidler *et al* 1960) and that the destruction of its nerve supply causes ultrastructural degenerative changes within 12 hrs (Farbman 1969) Active structures are in general very dependant upon their circulation It therefore seemed of interest to study the relation between the result of this activity *i e* the neural response to taste stimuli and the blood circulation. This series of experiments were therefore carried out in an attempt to shed some light on this relation

Methods

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as a blood plasma substitute

One femoral vein was always cannulated for i v injections and one femoral artery for recording blood pressure Both common carotid arteries as well as the external carotid arteries were dissected free to a point distal to the branching of the lingual arteries The external carotid arteries were tied off distal to this point The blood flow from the heart was arrested by either occlusion of the external carotid arteries or occlusion of both common and the external ones

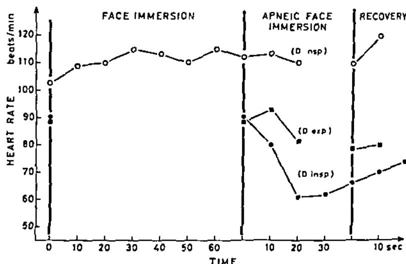


Fig 1 Heart rate during face immersion and breath holding in tracheostomized (0—0), and normal man, (●—●) prior to inspiration, (■—■) after expiration

Conclusion

It is our opinion that the slowing of the human heart during face immersion is at least partly, due to lack of movement of the thorax, and that the bradycardia is vagally induced as a result of peripheral vasoconstriction secondary to the breath holding (Cohn *et al* 1968, Blix & Myhre)

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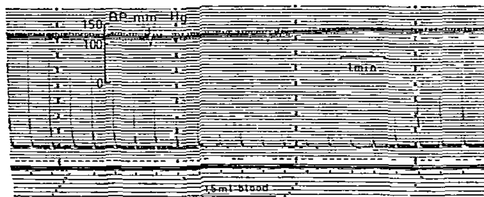


Fig 2 The record shows the deterioration and restoration of the summated chorda tympani response to 0 3 M NaCl in an animal without a simultaneous manipulation with the blood supply to the tongue It demonstrates that an i v infusion of 1 5 ml blood reversed the trend of the blood pressure recording and apparently restored the chorda tympani response Nembutal anesthesia

onset of each occlusion The moment of occlusion was asigned the value 0 Fig 3 indicates no consistent change in the rate of decay with increasing number of occlusions Similar conclusions could also be drawn from the results of other animals tested in this respect, provided that the taste receptors were given enough time for recovery and the occlusion did not last too long

The effect of restoration of the blood flow to the tongue

Fig 1 shows that the gustatory response returned when the occlusion was removed Fig 1 demonstrates also a stage of increased gustatory sensitivity before the summated response returned to the magnitude it had prior to the occlusion Similar increases of response were frequently observed especially at the beginning of an experiment and after occlusions which had lasted long enough to cause a complete or almost complete disappearance of the taste response In later stages of the experiments ex-

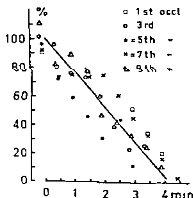


Fig 3 demonstrates the effect of repeated occlusion of the ipsilateral external carotid artery on the rate of decrease of the summated chorda tympani response to 0 3 M NaCl These responses were expressed in percentages of the average size of the last 5 responses obtained before the occlusion in question The moment when the occlusion was appl ed was asigned 0 The oblique line was fitted by eye The contralateral artery was left open Hypnorm anesthesia Flaxedil and artifical respiration



Fig 4 The figure shows the localization of the staining in three transverse sections of the tongue after intravital injection with stain through one lingual artery Hypnorm anesthesia

tended periods of inexcitability could be observed after an occlusion in connection with a lowered systemic blood pressure Intravenous infusions of blood or plasma substitute could then usually restore the taste response in a manner similar to that shown in Fig 2

The effect of bi contra and ipsilateral cessation of blood flow

No rat was able to maintain its gustatory sensitivity with both common carotid arteries occluded All rats were able to maintain their sensitivity if only their contra lateral common carotid artery was occluded Some animals were also to maintain their sensitivity for 15 min with their ipsilateral common carotid artery occluded tI is felt that the type of anesthesia played a role in this respect because the systemic blood arterial pressure was higher in rats which were under barbiturate anesthesia than under hypnorm In many cases under Hypnorm anesthesia no difference was observed between the rate decrease caused by bilateral or ipsilateral occlusion

Observations made after intravital staining through the lingual artery

Fig 4 may offer an explanation of the observation that occlusion of the ipsilateral artery usually abolished the gustatory response of the rats although their contralateral blood supply to the tongue had been left open Fig 4 shows the tongue of a rat sectioned transversally after intravital stain had been injected through one lingual artery Fig 4, which unfortunately cannot be reproduced in colour indicates that the staining was unilaterally localized Examination of the sections demonstrated that most staining could be observed close to the surface of the tongue In the tip of the tongue higher magnification showed small circular concentrations of blue colour probably in vascular plexuses especially under the fungiform papillae The tip of the tongue

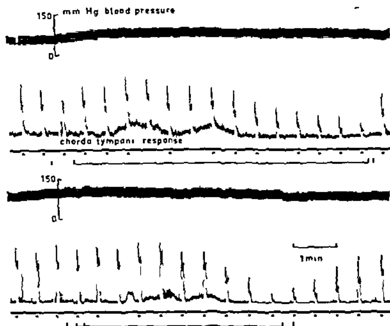


Fig 5 The records were consecutively obtained from the same animal during repeated stimulation with 0 3 M NaCl The blood flow to the tongue was in both records bilaterally

also showed blue staining close to its ventral median surface No staining at all was observed under the taste buds of the contralateral side of the tongue The back of the tongue was more uniformly stained, but there was still a more intense staining close of the surface The minor sublingual salivary glands, which are situated on each side of the tongue dorsal to the large veins were unstained This part of the experiments indicated that the taste buds were unilaterally supplied to a large extent It also indicated that the richest vascularization is beneath the taste buds

The effect of a simultaneous activation of the arterial chemo and baroceptor reflexes
It was observed that the rate of decrease of the gustatory response increased if a second clamp was applied around the common carotid artery before its division into the external and internal carotid arteries Fig 5 shows an example of this In this animal the external carotid arteries and the external and the common arteries were occluded in an alternative manner The lower recording of Fig 5 shows that the rate of decline increased when the blood supply to the baroreceptor and the chemoreceptor region of the carotid arteries also ceased In this animal the average times for a decrease to 25 % of the original value were 6 9 and 4 3 sec respectively The

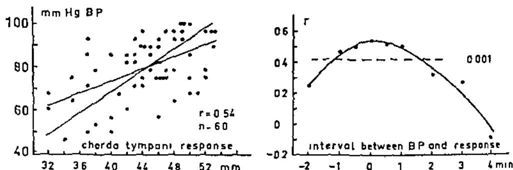


Fig 6 In the left graph the chorda tympani responses to 0 3 M NaCl obtained every half min were plotted against the simultaneously measured arterial blood pressure The oblique lines

difference was statistically significant ($p < 0\,001$) Similar consistent differences were obtained in other animals though these in general were smaller The existence of possible collaterals which may have interfered was investigated in two ways First the external carotid artery was dissected free from its origin to the point where it had been permanently occluded distal to the branching of the lingual artery Secondly colour was injected into the common carotid arteries after the lingual ones had been occluded No staining at all was observed in the tongue though other parts of the head became deeply stained

During the course of these experiments it became increasingly evident how close the relation is between the magnitude of the taste response and the circulatory status of the animal Fig 6 supports this suggestion Fig 6 a is based on data obtained during a 30 min uninterrupted recording period In Fig 6 a the chorda tympani response to stimulation of the tongue with 0 3 M NaCl every 30 sec was plotted against the simultaneously measured blood pressure The circulation status of the animal was affected through removal and infusion of 0 5 ml blood infusion of 1 ml Haemaccel removal and infusion of 0 8 ml blood and finally infusion of 0 2 ml blood The correlation coefficient between the chorda tympani response and the simultaneously measured blood pressure is 0 54 ($p < 0\,001$) The regression lines were included in Fig 6 a These data indicate that there was a rather close relation between the blood pressure and the chorda tympani response in this animal

The correlation coefficient between the blood pressure and the gustatory response measured at different time intervals was then calculated for the experiment described above and plotted in Fig 6 b Fig 6 b shows that the correlation was largest when there was no interval at all between these two parameters This indicates that they were both regulated or influenced by some superior mechanism as otherwise, if one of them had influenced the other, the largest correlation would probably have been observed at some delay

Discussion

The results of this study show that after cessation of the blood flow through the lingual arteries the response to taste stimuli recorded from the chorda tympani nerve will decrease and disappear completely within a few min. Further, they demonstrate that the taste response will disappear faster if the common carotid arteries are also occluded. Finally they indicate that during changes of the circulating blood volume the systemic blood pressure and the taste response recorded from the chorda tympani nerve varied simultaneously.

This discussion will attempt to identify the cause of the observations obtained by excluding some explanations which seem to be less probable.

First, we can conclude that accumulation of the stimulating substance after the cessation of the blood circulation cannot explain the decreased sensitivity, because the stimuli used did not penetrate the tongue (Mistretta 1968), but are rinsed away with water.

Secondly it may be concluded that the effects described can hardly be attributed to any effects on the nerve fibres of the deteriorated circulation. Observations from mechanoreceptors in which the non medullated part of the nerve serves as the transducer and the medullated as the mediator of the impulses elicited have not revealed a decrease of excitability after cessation of the circulation comparable to the one observed here.

It is therefore likely that the observed effects must be ascribed to changes of the taste receptor cell or to its relation with the gustatory nerve fibres.

For the further discussion, it may be of interest to compare some observations obtained in the study of another chemoreceptor, the arterial with some of those described for the gustatory. In general it can be suggested that the degree of the vascularization of a structure serves as a good measure of its metabolic rate. In the case of the arterial chemoreceptors, it is well known that carotid body is richly vascularized (*i.e.* De Castro and Rubio 1968). The gustatory receptors of the anterior part of the tongue of the rat are situated in the top of the fungiform papillae, in which region the i.a. injections made in this study produced the most intense staining. Consequently there are more vessels in this region of the tongue than in other. Farbman (1965) has also shown that these papillae contain several blood vessels. Fish, Malone and Richter (1944) remark that blue dots are observed at the top of these papillae after i.a. injection with blue colour. It may therefore be concluded that the taste buds are supported by a rich vascular network which indicates a high metabolic rate.

The circulation has a double task. It carries substances necessary for cell metabolism and removes metabolites. It seems impossible at present to conclude that failure to fulfil one or other of these tasks during the arrest of circulation was the main reason for the described effects on the taste receptors. For the arterial chemoreceptors the work by De Burgh Daly, Lambertsen and Schweitzer (1954) has shown a large oxygen consumption 9 ml O_2/min/100 g tissue. This figure is three times larger than that measured for the brain (Kety and Schmidt 1945) and of the

same order as that of the working left ventricle of the heart (McKeever, Gregg and Canney 1958) and demonstrates a large oxygen consumption for that kind of chemoreceptors

In the case of the gustatory chemoreceptors, observations obtained by Bradley (1970) have shown a strong dependance upon oxygen for the sensitivity of these cells He perfused the isolated rat's head with an emulsion of fluorcarbon FC 47 containing oxygen which kept the taste receptors viable But the taste response disappeared within 3 5—4 min if the oxygen was exchanged with nitrogen This figure is of the same order of magnitude as those which can be obtained in Fig 1 and 2 It seems from these various pieces of evidence that oxygen deficit caused by cessation of blood flow may be the cause of the gradual decrease of excitability described here

It is well known that changing the blood volume affects the autonomic nervous system A decrease of the circulating blood volume or clamping of the common carotid arteries elicits vasoconstriction in most organs (cf Chien 1967) in order to *maintain the blood flow to the most vital organs, like the brain and the heart* It is likely that the manipulations described above also affected the autonomic nervous fibres to the tongue which then contracted or dilated the vessels supplying the taste buds thereby changing their oxygen supply It is not unlikely that the amount of oxygen available for the taste cells was releated to the general vasoconstrictor tonus also when the blood flow to the tongue had ceased This explains the observations of Fig 5 and 6 The stage of increased sensitivity shown in Fig 1 was probably the result of a stage of reactive hyperaemia quite commonly observed after ischaemia However it is felt that some other factors like a direct efferent influence on the taste cells or a mechanical effect due to filling of the blood vessels beneath the taste buds which protruded them cannot be discarded as explanations for the data of Fig 5 and 6

Modification of the circulation to a receptor as a means of changing its sensitivity has been suggested for the arterial chemoreceptor by Eyzaguirre and Lewin (1961) There are also a few studies (Kimura 1961, Chernetski 1964) which indicate that the sensitivity of the taste receptors can be modified through the influence of the sympathetic part of the autonomic nervous system, though these authors have not pointed out the possible role of the circulation as the mediator

In summary this discussion indicates that the observed decrease of the taste response after the arrest of the blood circulation can be attributed neither to changes of the conductivity of the fibres outside the taste buds nor to an impaired removal of the tasted stimulus It suggests that the metabolism of the taste cells is high and that an inability to satisfy their demands during arrest of circulation may have caused the described inexcitability The observation of a simultaneous variation in blood pressure and the taste response suggests that the sensitivity of the taste cells in the rat can be altered through the influence of the autonomic nervous system

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From the Department of Zoophysiology, University of Oslo, Blindern Norway

Spatial Distribution of Receptor-Responses to Stimulation with Different Odours

By

HANNA MUSTAPARTA

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Abstract

MUSTAPARTA H *Spatial distribution of receptor-responses to stimulation with different odours* Acta physiol scand 1971 *82* 154—166

The olfactory receptor responses recorded from the *eminentia olfactoria* in the frog were studied to find whether receptors with different specificities were homogeneously or non homogeneously distributed Receptor potentials (EOGs) were recorded simultaneously from two different areas of the *eminentia olfactoria* 21 different substances were used 8 of these substances belonged to a homologous serie of cycloketones For each substance the ratio N between the two EOG amplitudes was measured N values obtained with the recording elec [illegible] ncen [illegible] the [illegible] lues [illegible] te a [illegible] s at [illegible] sted Keeping one electrode fixed and placing the other stepwise away in a posterior or lateral direction a successive general reduction in N values for all the substances was found This suggests a general reduction in receptor density in posterior and lateral direction

Olfactory discrimination is generally ascribed to the existence of different types of receptors Direct evidence supporting this view has been obtained by recordings from single receptors [illegible] in the olfactory epithelium of the frog (Gesteland *et al* 1963, Altner and Boeckh 1967 O'Connell and Mozell 1969) Results from these experiments showed that receptors could be selectively and specifically activated by different odours Gesteland *et al* found that many receptors were activated by all the 26 substances tested but exhibited high sensitivity to at least one odour No 2 receptors seemed to have identical patterns of sensitivity to the different odours Altner and Boeckh (1967) found receptors which responded to a small number of substances, although two out of the 28 substances used excited all the receptors tested Each of the 4 substances used by O'Connell and Mozell (1969) were found to excite about 50 per cent of the receptors tested

It is also possible that there is a spatial discrimination in terms of a differential distribution of receptors in the mucosa This idea was first proposed by Adrian

(1951) who found by recording from the olfactory bulb of the rabbit that lipid soluble and water soluble substances gave rise to greater activity in the anterior and posterior part of the bulb respectively This was assumed to be due to a spatial projection of the epithelium onto the bulb, which was confirmed histologically by Le Gros Clark (1951) These findings, together with the results obtained by Leveteau and MacLeod (1969) that single glomeruli in the olfactory bulb of the rabbit can be selectively activated gave rise to the question of the distribution of specific receptors in the olfactory epithelium

In most vertebrates including the rabbit, the receptors are distributed over extensive areas of a complex system of turbinates, and it is therefore difficult to explore their distribution In the frog however, the receptor region is easily accessible and recordings can be made under more favourable experimental conditions

The distribution of the different receptor types may be characterized as homogeneous or non homogeneous A homogeneous distribution implies that the receptor composition, *i e* the numerical proportion of the different receptor types is constant all over the epithelium The density may be constant or varying, but would in both cases give a constant relative activity for all substances at two compared areas The ratio N between two EOG amplitudes (Ottoson 1956, Müller, in press) recorded simultaneously would then remain unchanged for different substances

A non homogeneous distribution implies that the receptor composition is not the same in each unit area giving a variation in activity from one area to another because of variation in specific activity In this case the ratio N between two EOG amplitudes recorded simultaneously would vary for different substances

The present paper gives an account of a study of the receptor activity in different areas of the olfactory epithelium of the frog The results suggest that the receptor composition is non homogeneous

Material and Methods

Preparation The frog (*Rana temporana*) was anesthetized by urethane crystals applied to the skin fastened to a cork plate and maintained by artificial respiration The dorsal wall of the olfactory cavity on one side was removed exposing the central part of the olfactory mucosa with the *eminentia olfactoria*

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Stimulation The stimulating apparatus has been described previously by Drake *et al* (1969) and is shown in Fig 2 The stimulus consisted of 1 8 ml of a mixture of clean air and vapor
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magnet valve
allowed the air to pass through the vessel containing the odorous substance only during the stimulus period providing a constant air volume for stimulation

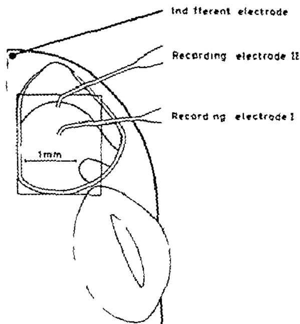


Fig 1 Schematic diagram of the frog head preparation showing the exposed area of *eminentia olfactoria* and *the location of the electrodes*

Since a succession of many 21 different odours at short (3 min) intervals was to be used it was necessary to have a suitable device which could ensure both a quick change of the stimulus and a constant outlet position. For this purpose a stimulating "carousel" was used. This consisted of a distributor and brass turntable on which 25 vessels were radially mounted each vessel containing one odour (Fig 3). By turning the table one could connect the particular vessel to the air passage from the humidifier. The air could then be passed over the odorous solution and through the outlet which had the form of a capillary 2 mm in diameter. The capillary was positioned about 3 cm over the epithelium. Because of this arrangement and the relatively small size of the *eminentia olfactoria* (approx. 3 mm²) the stimulus was assumed to

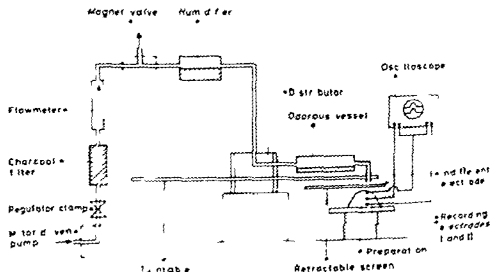


Fig 2 Schematic illustration of the stimulating and recording arrangement

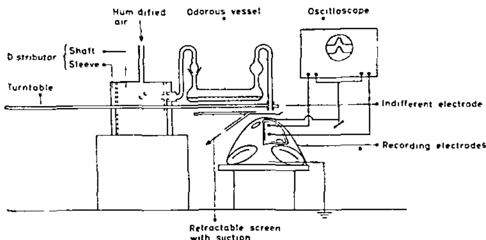


Fig 3 Illustration of distributor, odorous vessel and the retractable screen with suction The latter prevents the odorous air reaching the epithelium during the first second after the air is switched 'on" (see text)

be evenly distributed over the epithelium A retractable screen was placed over the epithelium and connected to a suction device and to the magnet valve in such a way as to prevent odorous air from reaching the epithelium before the saturated odorous air was blown onto the epithelium

The following stimulating substances were used

Group A A homologous series of cycloketones with 4 to 12, and 15 carbon atoms C_4—C_{12} and C_{15}

Group B A group of non homologous substances Amyl acetate (acetic acid pentyl ester), camphor, propanol, β ionone, benzene, heptanol, decanol, nitrobenzene, coumarin, naphthalene and pyridine

The substances were dissolved in diethylphthalate and 2 ml of each solution was pipetted into its respective vessel on the carousel One of the vessels was filled with pure solvent and used as blank to test for mechanical artifacts or impurities in the air

shifted while the other was kept at its original position and the same substances retested In this way by varying only one electrode position, EOGs from several recording fields could be compared with the EOGs from the stationary field In some experimental series the 'variable' electrode was placed at successively increasing distances on a straight line away from the 'stationary' electrode The stationary electrode was usually placed at the anterior part of *eminentia olfactoria*

Each experimental series took about one and a half hours During this time the recording conditions may be disturbed by physiological and electrical changes To test for possible changes in experimental conditions the first stimulus was repeated at the end of each experimental series

Statistical treatment The treatment of the results is based on the assumption that no appreciable systematic change in recording conditions occurred during the experimental series To test this assumption the first stimulus was repeated at the end of each run as described above Thereby two values of ratio V the former V_F and the latter V_L were obtained for the same stimulus tested at the same positions The difference between V_F and V_L could be due to systematic errors i e changes in recording conditions and/or to random variations in the measurement of the amplitudes The repetition of the first stimulus was made for 54 runs and the difference $d_i = V_{F_i} - V_{L_i}$ calculated Thereby 54 e

If the difference $d_i = N_{F_i} - N_{L_i}$ were due only to random variation and no appreciable errors had occurred the expected value of d_i would be equal to zero, $\bar{d} = 0$, and the variance

$$s^2 = \frac{1}{n-1} \sum_{i=1}^{n} (d_i - \bar{d})^2$$

Assuming a normal distribution of d_i it is possible to calculate the so-called tolerance limits i e, the limits within which a specified percentage of the present population of d_i should lie. The 99 per cent tolerance limits for 53 degrees of freedom is $\bar{d} \pm 3.1 \times s$ (Owen 1962) The 54 d_i values give the mean value of d_i, $\bar{d} = 0.4 \times 10^{-2}$, variance $s^2 = 48.3 \times 10^{-4}$, standard deviation $s = 7.0 \times 10^{-2}$ Thus the lower limit is -0.231 and the higher 0.213 Since the one relatively high value of $d_i = 0.20$ also lies within these limits, there is no reason to suspect that any appreciable systematic error has occurred during the experimental series Therefore, the above assumption may be considered as well confirmed

Results

All the EOGs recorded were monophasic and negative — going with amplitudes varying from 0.5 to 3 mV With equal molar concentrations (1, 10^{-1}, 10^{-2}) amyl acetate camphor C_9, C_{10} C_{11} and C_{12} gave higher amplitudes of EOGs than the lower cycloketones (C_4, C_5, C_6, C_7 and C_8) and the other substances in Group B Coumarin and cyclopentadecanone gave amplitudes too small to measure at the dilution used All the other substances at the dilutions used produced responses at each recording field tested within the sensory epithelium Different substances showed characteristic configurations of the EOG as previously reported by Ottoson (1956, 1958)

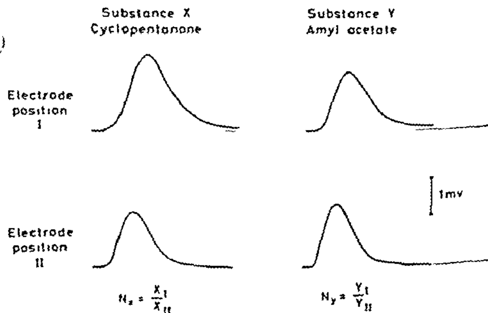


Fig 4 Simultaneous EOGs for two odours obtained at the recording positions I and II, giving the EOG amplitudes X_I, Y_I for position I and X_{II}, Y_{II} for position II Sweep length is 14 sec

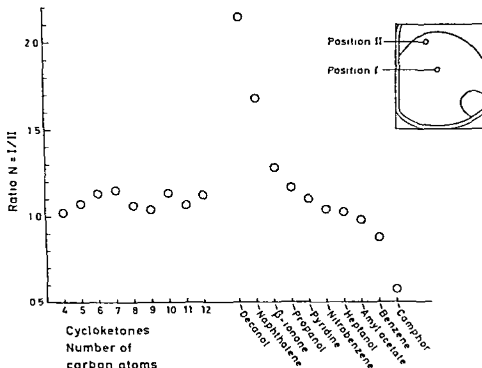


Fig 5 Ratio N for different odours obtained by comparing the EOG amplitudes from the recording position I and II

The two EOG-amplitudes obtained for each stimulus were measured and the ratio N calculated An example is given in Fig 4, which shows records of responses to cyclopentanone (X) and amyl acetate (Y), at two recording positions, I and II The amplitudes X_I, X_{II}, Y_I and Y_{II} result giving the ratio $N_X = \frac{X_I}{X_{II}}$ and $N_Y = \frac{Y_I}{Y_{II}}$ for the two substances X and Y

In Fig 5 to 9 the ratio N is plotted for different substances with one or two concentrations at various electrode positions Table I shows the range of N obtained from experiments where electrode I was placed at positions of successively increasing distances from electrode II The range for each experimental series is calculated as maximum relative range $t = N/\bar{N} \cdot 100$ where N is the range $N_{max}—N_{min}$ and N is the arithmetic mean of these two extremes For each experimental series a relative range of N is shown in Table I for all the substances in Group A and B together and for the substances in Group A alone

1 *Variation in the ratio N for different stimulus qualities at the same recording position* In most experimental series the ratio N was observed to vary for different stimulus substances An example is shown in Fig 5 where the N-values are plotted

TABLE I The range values obtained for 2 groups of substances at increasing inter electrode distances

Distance between electrode I and II mm	Maximum relative range $t = N/\bar{N}$ 100 for all substances in Group A and B and for only the Group A substances															
	Experiment 1		Experiment 2		Experiment 3		Experiment 4		Experiment 5		Experiment 6		Experiment 7		Experiment 8	
	A+B	A	A+B	A	A+B	A	A+B	A	A+B	A	A+B	A	A+B	A	A+B	A
0.08	25	10	18	12	16	6	18	13	26	15	18	18	27	12	26	10
0.15	35	13	21	6	38	7	43	6			16	8				
0.23	37	13	29	15			18	32	11	11			26	9		
0.30			51	7	16	10					47	5			46	12
0.38							32	1	13	7			72	15	43	16
0.45					30	14					65	13				
0.53							38	30	24	28					48	7
0.60											26	26				
0.68							31	25	25	15			96	20	29	19
0.75											39	4				
0.83															52	23
0.90									53	18	43	3	81	24		
0.98																
1.05									34	15						

for one series of 19 different odours Relatively large variations in N values are observed for some substances from camphor (0.5) to decanol (2.15) Within the homologous series of cycloketones the ratio N is almost the same (1.03—1.15)

For the experimental series shown in Table I there can also be seen relatively low range values for the cycloketones compared to those obtained for all substances

2 Variation of the ratio N for different electrode positions and the same stimulus When shifting electrode I from one position to another while keeping electrode II fixed alterations of N values were observed for each of the stimuli Fig 6 shows an example where ratio N is plotted for two electrode positions I_1 and I_2 The N-values for cycloketones are lower at position I_2 than at position I_1 For propanol pyridine naphthalene and benzene however the values of N are higher at position I_2 than at position I_1

In most experiments the N-values for the cycloketones decreased when electrode I was shifted from an anterior to a posterior or lateral position This decrease was not always found for the substances in Group B

3 Variation in ratio N for different substances with increasing inter electrode distances In some experiments electrode I was placed successively further away from electrode II With a distance of 0.08 mm between the electrode centers the N values were close to 1 while at greater distances the ratio showed considerable variations It was also found that a general decrease in N values occurred when electrode I was placed in a posterior or in a lateral direction

Fig 7 shows the results from an experiment in which electrode I was shifted in a posterior direction The N values are plotted for 3 substances only propanol

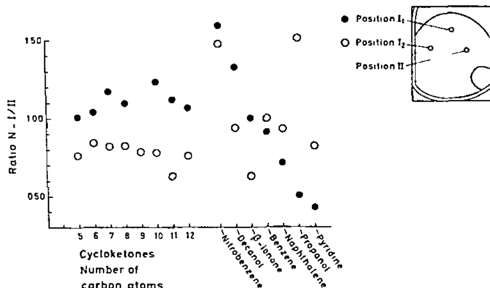


Fig 6 Two sets of N values obtained for 15 odours at the two recording positions I and I_2 of electrode I while electrode II was kept fixed

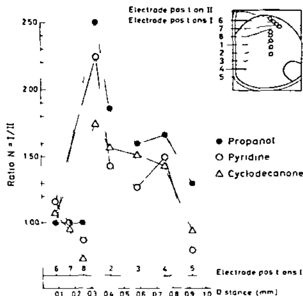


Fig 7 Eight sets of N values shown for three substances, propanol, pyridine and cyclodecanone Each set is obtained by shifting electrode I to eight different positions, five of which are lying at successively increasing distances from electrode II in posterior direction

pyridine and cyclodecanone The cycloketones and some substances in Group B showed N-values similar to those of cyclodecanone The values obtained for propanol and pyridine differed the most from those for the other substances In the diagram it can be seen that the N values lie close to 1 for position I_6, which has a distance of 0 08 mm from position II The positions I_2 I_3, I_4 and I_5 having a greater distance

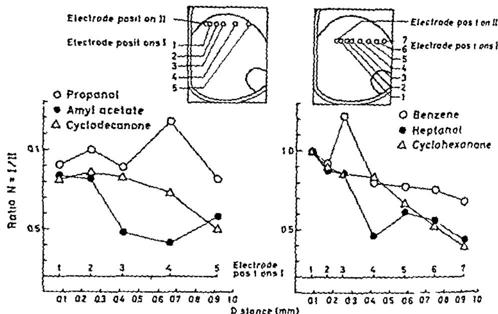


Fig 8 N values obtained by placing electrode I in lateral direction The po itions in the left diagram lies 0 30 mm anterior of those in the right The left and right diagram include five and seven sets of N values respectively for three substances

from position II show a greater variation in N values for different substances For example position I_1 with a distance of about 0 30 mm shows a range of N values from 2 50 to 1 75 while position I_6 with only 0 08 mm distance shows a range of 1 16 to 1 00 In addition marked reduction in N values for all the substances from position I_1 to I and from positions I_j to I_8 I_7 and I_8 was found The positions from I_1 to I_5 are successively farther away in a posterior direction from electrode II The positions I_6 I_7 and I_8 are quite anterior and lateral on the *eminentia olfactoria* on the downward sloping side

Fig 8 shows N values obtained from 2 expts where electrode I is placed successively further away in a lateral direction The values plotted in the right diagram were made 0 30 mm posterior to those in the experiment shown in the left diagram

In these diagrams cyclodecanone and cyclohexanone represent the majority of the substances studied Propanol acetate (left) benzene and heptanol (right) are substances which showed the greatest deviation It can again be seen that positions I_1 in both diagrams with a distance of 0 08 mm from position II show N values close to 1 A marked increase or decrease in N occurs first at a distance of 0 23 mm from position II and the variation is greater for each of the following positions For example while position I_1 left shows a range of 0 90—0 81 position I_4 at a distance of 0 68 mm from position II shows a range of 1 18—0 41 Both diagrams also show an average decrease of the N values in lateral direction as for cyclohexanone from 1 00 at position I_1 to 0 39 at position I_7

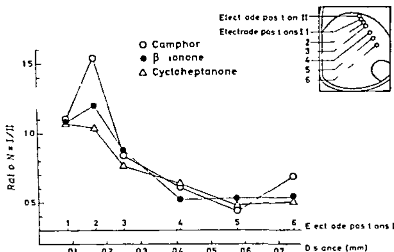


Fig 9 Six sets of N values for three substances obtained by shifting electrode I to six different positions in a posterior lateral direction

In Fig 9 are shown N values from positions posterior lateral to and increasingly distant from position II Cycloheptanone is chosen to represent substances showing fairly constant N values while camphor and β ionone represent substances showing greater deviations Position I_1, 0 08 mm distant from position II, again shows N values close to 1, which implies a small range In this diagram it is only position I_2 with a distance of 0 15 mm from position II, which shows a considerable range of 1 53—1 03 A decrease in N values from position I_1 to I_6 in the posterior lateral direction is shown for all the substances

The results have been summarized in Table I which shows the maximum relative range of N for each position corresponding to different distances from electrode II The smallest distance 0 08 mm shows a relative range of about 20 for all the sub stances Further away this value can be as high as 96 although relative range is not directly correlated with increasing distance The relative range of N for the sub stances in Group A varies from 1 to 30 In this group the range values are not necessarily smaller at 0 08 mm than at greater distances

4 *Variations of the ratio N with different concentrations of one and the same odour at the same position* In some experiments two or three concentrations of the same substances were tested At some positions the ratio N changed with the con centrations Examples from experiments carried out for cycloketones in 1 M 0 05 M and 0 1 M concentrations are shown in the Fig 10 In these diagrams the ratio N is plotted for two concentrations of each odour at one position High concentration is 1 M for C_5—C_8 0 5 M for C_9—C_{10} Low concentration is 0 5 M for C_5—C_8 0 1 M for C_9—C_{10} For all the substances in Fig 10 a N is lower at the lower con centrations than at the higher concentrations For substances in Fig 10 b N is higher at lower concentrations than at the higher ones

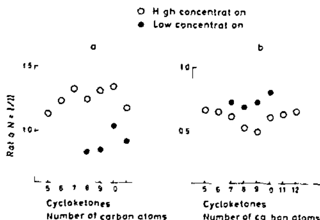


Fig 10 N values obtained for two concentrations of some odours when electrode I and II were kept fixed

a The N values of the low concentration are lower than those for the high concentration

b The N values of the low concentration are higher than those for the high concentration.

Discussion

The results in Part 1, showing that the ratio between two EOG amplitudes varied for different odours support the hypothesis of a non homogeneous distribution of different receptor types By placing one electrode at different positions while the other remained fixed responses arising from areas of the same size could be compared as shown in Part 2 These areas showed different N values for the same stimulus The decrease in N values for all the cycloketones from position I_1 to I_* (*cf* Fig 6) may be due to a general reduction in receptor density from recording field I_1 to I For propanol pyridine and naphthalene however the N values are lower at positions I_1 than I indicating that not all types of receptors are equally reduced in number at the recording field I These findings indicate that the variations in N values found from one position to another are not due to variations in receptor density alone It is more likely that areas equal in size, lying at a distance from each other are dissimilar both in composition and density of receptors

Part 3 describes recordings of EOGs from areas at successively increasing distances from each other Assuming that the tested areas were sufficiently small in size one would expect that areas lying close or adjacent to each other would have a greater similarity in receptor composition than more distant areas In Fig 7, 8 and 9 the N values for all substances lie close to 1 giving a comparatively small relative range when recording electrodes I and II are placed 0 08 mm from each other This indicates that the responses recorded arise in areas with similar receptor compositions and densities which may be due either to the fact that the recording fields overlap each other or that the area surrounding the two electrodes has a relatively homogeneous distribution of receptors The increase in relative range observed by increasing the distance between the electrodes indicates that areas lying at a greater distance from each other have greater differences in receptor composition

An estimation of the size of the recording field is of importance in interpreting the present results Information about the size of the recording field is obtained from

experiments carried out as described in Part 3 When the distance between the two electrodes was increased one would expect a gradual diminished overlap of their recording fields This should give a gradual increase in range values, since the receptors are non homogeneously distributed The range values in Table I are always relative small for the initial step (0.08 mm), but do not increase successively with the stepwise distance increase of 0.08 mm This indicates that the recording fields only overlap to a considerably degree within a distance of 0.008 mm between the electrodes and are too small in relation to the stepwise increase of 0.08 mm to give a successively diminished overlap These findings may agree with results from experiments reported by Daval, Leveteau and MacLeod (personal communication) They found when stimulating the epithelium electrically that the response amplitude decreased to 1/e at a distance of 400 μ from the stimulaing electrode

The variations of ratio N for the cycloketones are small compared to those for Group B, even though the distances between recording points are large This suggests that the cycloketones have similar stimulating properties However, the results in Part 4 show that the ratio N varies with different simulus intensity for a given substance In this connection it must be mentioned that the lower cycloketones (C_4—C_8) were used at a concentration of 1 M and the higher (C_9—C_{12}) at 0.05 M as strongest solution At these concentrations the N values for all of the substances (C_4—C_{12}) lay within a narrow range in each experimental series and the same was found for the solutions ten times diluted from these original concentrations Camphor and amyl acetate were used at a concentration of 0.1 M as strongest solutions and the other substances at 0.5 M or 1 M The objection can be raised that it would be possible to choose for the B group substances concentrations giving equal N values at one position However by shifting the electrode to another position and using these specially chosen concentrations, the N values might vary in different degrees for each substance as shown in Part 2 For cycloketones however, a small range was found for all positions examined

Assuming a non homogeneous receptor distribution the considerable decrease or increase in N values shown for different concentrations of the same substance in Fig 10 may be explained by higher or lower thresholds of receptors in the areas recorded from Another possibility may be that the receptor types give different increase in response to the same increase in intensity for a given odour as found by O Connell and Mozell (1969) in recordings from single receptors in the epithelium of the frog The curves obtained by plotting the impulse responses against the concentrations of a given substance showed different gradients for different units

However, the recorded variations in ratio N for different concentrations seem not to agree with the results obtained by Mozell (1966) He found that the ratio between amplitudes measured at the medial and lateral nerve branches from the olfactory epithelium of frog showed small changes with different concentrations and the four chemicals used were equally ranked in all experiments independent of concentrations A possible explanation is that these medial and lateral areas on the epithelium have a relative similar receptor composition

The results obtained have been interpreted according to the assumption that there exists different types of receptors. Another mechanism for discrimination has been postulated by Mozell (1964, 1966) who assumes that the mucus layer acts as a gas chromatograph separator and thereby enhances discriminating power. It is difficult to design experiments to test this hypothesis. If the recorded difference in activity depended entirely on selective separation by the mucus layer, one would expect the ratio N to remain constant at different concentrations. However, as has been shown the N-value may vary with different concentrations of one and the same substance. These variations could not be caused by the separator mechanism alone but rather by a selective sensitivity and non homogeneous distribution of receptors or possibly a combination of these two mechanisms.

Models of the discriminating mechanism assume convergence of the neural pathways from receptors to secondary neurons and from secondary to tertiary etc. It may be possible that the *secondary neurons in the olfactory bulb are activated from* more-or less limited areas in the epithelium. If the limitation of these areas is dependent on the non homogeneity in receptor distribution the areas activating several secondary neurons would overlap each other to different degrees depending on the distribution pattern of the receptors. In this way a non homogeneous distribution of the different receptors might simplify the convergence pattern thereby facilitating the sorting of the information from receptors to the secondary neurons.

I would like to thank Dr. Kjell Døving for suggesting the topic and for his constant encouragement during the work.
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From the Department of Geriatrics University of Uppsala and King Gustaf Vth Research Institute Stockholm Sweden

Effect of Prolonged Strenuous Exercise on the Concentration of Triglycerides, Phospholipids and Glycogen in Muscle of Man

By

SVEN O FROBERG and FOLKE MOSSFELDT

Received 26 Oktober 1970

Abstract

FROBERG S O and F MOSSFELDT *Effect of prolonged strenuous exercise on the concentration of triglycerides phospholipids and glycogen in muscle of man* Acta physiol scand 1971 82 167—171

— Muscle tissue from the lateral vastus of the femoral muscle was taken by needle biopsy The uring ogen The results indicate that not only muscle glycogen but also muscle triglycerides are of importance for the energy metabolism in man during exercise

During exercise energy is derived both from fat and carbohydrates The plasma free fatty acids have been shown to be an important source of energy during exercise (Miller Issekutz and Rodahl 1963 Issekutz *et al* 1964 Paul and Issekutz 1967 Havel Naimark and Borchgrevink 1963 Havel *et al* 1964 Carlson Froberg and Persson 1965) In man however immediate oxidation of plasma free fatty acids accounted for less than 50 per cent of the CO_2 produced during exercise at an RQ of 0 75 (Havel *et al* 1963 1964) This has led to the assumption that also fatty acids derived from muscle stores of triglycerides were oxidized during the exercise Direct studies on muscle during exercise are however controversial In bird wing muscle exercise has been reported to decrease (Georg and Jyoti 1955) or to increase (Vallyathan Grinyer and George 1970) the amount of neutral fat while in mammalian gastrocnemius muscle exercise was without effect on the concentration of esterified fatty acids (Buchwald and Cori 1930—31 Cemmill 1940 Masoro *et al* 1966)

The following is a report on the effect of strenuous exercise on the concentration of triglycerides and phospholipids in the femoral muscle of man The exercise consisted of cross country skiing at high metabolic rate for about 7 hrs

TABLE I Anthropometric data of the subjects Work capacity at a heart rate of 170 (W_{170}) on the bicycle ergometer was determined 4 days before the skiing

	Age Years	Height cm	Weight kg	W_{170} kpm
M	35	181	71	1656
SEM	4	1	2	96
Range	24—53	177—186	66—79	1300—1920

W_{170} = workload at a heart rate of 170
M = mean
SEM = standard error of the mean

Subjects and Methods

7 healthy male volonteers were studied They participated in a skirace of 85 km over hilly
terrain (Vasaloppet 1969) Table I gives some anthropometric data of the subjects No major
difference was observed in height and weight of the participants, the age varied considerably
as did also the work capacity at a heart rate of 170 (W_{170}) which was determined on a bi
cycle ergometer 4 days before the race The mean workload at a pulse rate of 170 was 1656
kpm/min

Muscle samples were taken before the race between 5 and 6 a.m after an overnight fast.
Then the subjects had breakfast During the race which started at 7 45 a m the subjects
had free access to blueberry soup at ten stations The blueberry soup contained about 30 %
sugar and in total about 400 g of glucose was consumed by each skier The sampling of muscle
after the exercise was done 24 ± 5 min after cessation of the skiing The heart rate was deter
mined at 3 occasions after uphill at level and after downhill terrain

Muscle was taken by needle biopsy technique from the lateral vastus of the femoral muscle
(Bergstrom 1962 for lipid determination Before exercise muscle tissue was taken from both
legs After the exercise muscle tissue was taken from both legs in 3 subjects and from one
eg in 4 subjects The glycogen concentration was determined in only one leg both before and
fter the exercise on an aliquot of a biopsy specimen used for lipid determination

The skin was anesthetized with 1 to 2 ml of 1 per cent Citanest® (Astra) about 5 min before
a skin incision of about 3 mm was made to facilitate the introduction of the biopsy needle
(diameter 5 mm Muscle tissue for lipid determination was dissected free of visible fat
and connective tissue and 20 30 mg was homogenized into methanol not later than 5 min
following the biopsy Two ml of chloroform was then added to the methanol homogenate fol
lowed by 3 ml of saline The concentration of phospholipids and triglycerides was determined
on aliquots of the chloroform phase as earlier described Froberg 1967) About 10 mg of
muscle tissue for glycogen determination (Hultman 1967 was homogenized into 1 2 ml of
9 per cent TCA solution not later than 7 min after the biopsy was taken
Statistical analysis was done according to Snedecor (Snedecor 1961)

Results

The mean time used for skiing was 447 min and the mean speed during the skiing
was 11 5 km h The mean heart rate was 156 beats/min (Table II) The heart rate
was determined from three registrations only and is probably too crude to estimate
the mean heart rate during the 7 hrs race

Table III gives the individual values for the triglyceride concentration in muscle
before and after exercise No precaution was taken to separate between samples
from right and left legs when muscle tissue from both legs was analysed In a previous
study, however no difference was found in the lipid content between right and
left legs either before or after exercise Carlson, Eklund and Froberg 1970 The

TABLE II Mean heart rate, duration and mean speed during the skiing

	Heart rate beats/min	Duration of skiing min	Mean speed km/h
M	156	447	11 5
SEM	3 2	17	0 4
Range	139—167	402—524	9 7—12 7

M = mean
SEM = standard error of the mean

absolute value for the differences between the legs before exercise in the present study (mean ± SEM) was 6 40 ± 3 0 μmoles/g When values on the triglyceride con centration were obtained from both legs a mean value was calculated and used in the statistical evalution on the effect of exercise During the exercise the mean triglyceride concentration decreased from 16 95 to 8 27 μmoles/g wet weight (Table III IV) The individual decrease was 8 68 ± 1 78 μmoles/g ($p < 0\ 005$) This corresponds to a decrease of 50 ± 6 per cent In two subjets the triglyceride concentra tion in one leg was unusually high before the exercise (Table II) This may have been due to contamination of these tissue pieces with adipose tissue If these values were omitted in the calculations the average individual decrase became 6 16 ± 1 23 μmoles/g wet weight ($p < 0\ 005$) corresponding to a decrease of 43 ± 7 per cent

No change occurred in the phospholipid concentration during exercise (Table IV)

The mean glycogen concentration calculated on the basis of wet weight decreased from 15 89 to 6 99 mg/g wet weight (Table IV) The individual difference was 8 89 + 1 66 mg/g ($p < 0\ 005$) which corresponds to a decrease of 55 ± 4 per cent

TABLE III Individual data for the triglyceride concentration, μmole g in the vastus lateralis of the femoral muscle before and after exercise

	Before exercise			After exercise			Indiv diff of means before after exercise	% dec rease
	1[a]	2[a]	Mean	1[a]	2[a]	Mean		
	36 33	15 63	25 98	—	13 40	13 40	12 58	48
	12 08	9 71	10 90	3 80	5 08	4 44	6 46	59
	28 87	14 27	21 55	6 36	5 06	5 71	15 84	74
	13 24	10 01	11 63	—	9 0	9 0	2 63	23
	23 74	20 81	22 28	8 80	12 55	10 68	11 60	52
	10 84	10 46	10 65	—	4 60	4 60	6 05	57
	15 97	15 30	15 64	—	10 05	10 05	5 59	36
M			16 95			8 27	8 68	50
SEM			2 38			1 30	1 78	6
							<0 005	

M = mean SEM = standard error of the mean
[a] indicates the triglyceride concentration in muscle samples from different legs No difference was made between right and left legs

TABLE IV. Mean concentration of triglycerides, phospholipids and glycogen in the vastus lateralis of the femoral muscle before and after exercise and mean of individual differences before and after exercise

	Triglycerides µmole/g			Phospholipids mg/g			Glycogen mg/g		
	B	A	Indiv diff B—A	B	A	Indiv diff B—A	B	A	Indiv diff B—A
M	16.95	8.27	8.68	9.57	9.31	0.27	15.89	6.99	8.89
SEM	2.38	1.30	1.78	0.66	0.40	0.47	2.48	1.03	1.66
			<0.005			>0.05			<0.005

B = before exercise A = after exercise
M = mean SEM = standard error of the mean

Discussion

After the exercise the concentration of triglycerides as well as of glycogen in the vastus lateralis of the femoral muscle was markedly decreased.

The decrease in the muscle triglyceride concentration is compatible with the hypothesis of increased utilization of endogenous muscle triglyceride fatty acids in fasting man during exercise (Havel *et al.* 1963, 1964). The lack of net decrease in the muscle concentration of phospholipids suggests that phospholipids, which are mainly a structural lipid fraction, were of no major importance in the energy metabolism, *e.g.* as an additional endogenous source of fatty acids besides the muscle triglycerides.

The effect of exercise on muscle lipids has previously been studied in muscle made to contract by electrical shocks. In bird the neutral lipid content has been reported to decrease (George and Jyoti 1955) or to increase (Vallyathan, Grinyer and George 1970) mainly in the red fibres (George and Jyoti 1955) of the exercised wing muscle. The decrease in the lipid content occurred in wing muscle fatigued after less than 30 min of exercise, the increase after up to 5 hrs of exercise. In monkey (Masoro *et al.* 1966) and rat (Gemmill 1940, Buchwald and Cori 1930—31) gastrocnemius muscle analysed for lipids without regard to fibre types, exercise was however without effect on the concentration of esterified fatty acids. The different results on the muscle lipid concentration after exercise may to some extent be due to species differences with regard to the metabolism of endogenous fatty acids during exercise. Other factors such as work intensity and duration of work may also have influenced on the rate of utilization of esterified fatty acids in muscle. In fact during bicycle exercise to exhaustion the muscle triglyceride concentration of the femoral muscle decreased 25 per cent (Carlsson, Ekelund and Froberg 1970). The disappeared muscle triglyceride fatty acids were estimated to account for about 70 per cent of the fat oxidized per minute during the exercise.

In the present study the mean concentration of triglycerides as well as of glycogen in the femoral muscle decreased about 50 per cent. The decrease in the muscle glycogen concentration during the skiing is in accordance with previous findings after bicycle exercise (Ahlborg *et al.* 1967, Bergström *et al.* 1967, Hermansen, Hultman

and Saltin 1967) The present data permit no conclusion about the quantitative role of the endogenous muscle substrates in the total energy metabolism during the skiing The values in Table IV may, however be used to estimate the amount of energy contributed by the endogenous substrates in the muscle studied, *e g* per gram wet weight of muscle tissue obtained in the biopsy needle If due to complete oxidation the disappeared triglyceride fatty acids correspond to about 65 cal and the disappeared glycogen glucose to about 35 cal *e g* in the muscle tissue studied oxidation of triglycerides accounted for about twice as many calories as did oxidation of glycogen

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Acta physiol. scand. 1971. 82. 172—176

From the Departments of Surgery, Neurosurgery A, Roentgenology and Clinical Neurophysiology
University Hospital, Lund, Sweden

Persistence of Cerebral Blood Flow Autoregulation Following Chronic Bilateral Cervical Sympathectomy in the Monkey

By

B. EKLÖF, D. H. INGVAR, E. KÅGSTRÖM and T. OLIN

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Abstract

EKLÖF, B., D. H. INGVAR, E. KÅGSTRÖM and T. OLIN. *Persistence of cerebral blood flow autoregulation following chronic bilateral cervical sympathectomy in the monkey.* Acta physiol. scand. 1971. *82.* 172—176.

The cerebral blood flow was measured in 11 monkeys after step changes in arterial blood pressure in the pressure range 30—140 mmHg. In one group of 5 animals a bilateral excision of the *superior cervical ganglia was performed about 2 weeks prior to the experiment.* These animals as well as those with an intact sympathetic innervation of the cerebral vessels, demonstrated an almost constant rCBF at pressures above 50—60 *mmHg, i.e.* both groups showed autoregulation. *It is concluded that cervical sympathectomy does not abolish autoregulation of the cerebral blood flow.*

The concept of a nervous (vasomotor) control of the cerebral circulation has had many advocates (*e.g.* Forbes and Wolff 1928) and opponents (*e.g.* Bayliss and Hill 1895); in the past Fog (1934) concluded that the active regulation of the cerebral circulation which he observed on the pial surface following variations of the blood pressure represented an autoregulation. Lately Falck *et al.* (1965) have shown an abundance of adrenergic fibers around the cerebral vessels. In 1969 James *et al.* reported that acute unilateral cervical sympathectomy abolished autoregulation of the cerebral blood flow, a finding which would assign a very fundamental role to the adrenergic innervation of the blood vessels of the brain. Since, however, numerous clinical and experimental studies (cf. Lassen 1959) have failed to show any definite *importance of these fibers for the cerebral circulation,* it was decided to study the effects of chronic bilateral cervical sympathectomy on the autoregulation of the cerebral blood flow.

Methods

Altogether 18 rhesus monkeys were studied, but 7 have been excluded from the present analysis due to ventilator instability which gave fluctuations in P_aCO_2.

6 monkeys (controls, average weight 2.5 kg) were anesthetized with Pentalsodium [illegible] or Halothane®. [illegible] and tracheotomized. After ligation of the right external carotid

artery, a fine radiopaque catheter was placed in the right carotid artery via the right femoral artery for injections of ^{133}Xenon solution. The position of the catheter was checked by cerebral angiography in each case. Ventilation was maintained with 70 % N_2O and 30 % oxygen. Flaxedil® (Rhodia) was used intermittently as muscle relaxant. The P_aCO_2, P_aO_2 and blood

levels of decreased MAP.

In another group of 5 rhesus monkeys (average weight 2.4 kg), bilateral resection of the superior cervical sympathetic ganglia was made under i.v. Brietal sodium® anesthesia. About two weeks later they were anesthetized with Halothane® and subjected to the same procedures and rCBF studis as the controls.

Results

At a MAP of 100 mm Hg the mean P_aCO_2 in the control series was 35.7 mm Hg and in the sympathectomized series 34.6 mm Hg. The respiratory conditions therefore appear to have been approximately identical in the two groups. The mean rCBF at that pressure level was 69.8 ml/100 g/min in the controls and 48.8 ml/100 g/min in the sympathectomized animals. There were differences in mean rCBF between individual animals in both groups most likely due to unavoidable variations in depth of anesthesia and hence, in cerebral metabolic rate, but the P_aCO_2 was kept reasonably constant in each single animal. For comparison of rCBF values we have expressed flows in per cent, where 100 % represents the flow measured at a MAP of 100 mm Hg (Fig. 1). In the controls the rCBF within the pressure range 140—60 mm Hg was practically constant. Lowering the pressure to values below 60 mm Hg the expected fall in rCBF was recorded (Table I). Following a ventilatory disturbance which provoked anoxia for 10 min in one of the animals (excluded from the series),

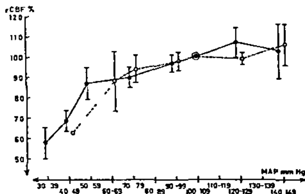


Fig. 1. Mean rCBF in per cent of value at mean arterial pressure 100 mm Hg. Summary of rCBF determinations with standard deviations in different MAP ranges. Open circles represent control animals and filled circles represent sympathectomized animals.

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From the Departments of Surgery, Neurosurgery A, Roentgenology and Clinical Neurophysiology
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Methods

Altogether 18 rhesus monkeys were studied but 7 have been excluded from the present analysis due to ventilator installation which gave fluctuations in P_aCO_2

6 monkeys (controls average weight 2 5 kg) were anesthetized with Brietal sodium® (Lilly), or Halothane® [illegible] and tracheotomized After ligation of the right external carotid

as in the controls was also seen (Table II) In some animals a slowing of the EEG was seen when the lowest flow values were recorded and in this respect as well as in others there were no conspicuous differences in the EEG records between the two groups

Discussion

The experiments have confirmed a good autoregulation of the cerebral blood flow when the MAP changes (Fog 1934, Haggendal 1965 Harper 1965, Lassen 1966) Siesjo and Zwetnow (1970) measuring metabolites in the cerebrospinal fluid and brain tissues during graded hypotension did not find any significant changes in the energy metabolites until the MAP fell below 40 mm Hg They registered however, a gradual increase in the brain tissue lactate concentration even at a moderate hypotension The speed of the autoregulatory response has been studied by Ekstrom Jodal and her associates (1970) They found that within the autoregulatory pressure range, CBF first changed in the same direction as a given pressure change but then gradually returned to its basal level within 45 sec In order to measure rCBF during steady state we therefore waited for 10 min following a change in arterial pressure In one animal the postanoxic loss of autoregulation and uncoupling of the relationship between rCBF and EEG frequency was shown with a re establishment of this relationship and the autoregulation after some hours (Freeman and Ingvar 1968)

The influence of the sympathetic nervous system on the autoregulation of the cerebral blood flow has been vividly discussed lately Falck *et al* (1965) have shown with their fluorescence technique an abundant nerve net of nor adrenalin containing fibers in the adventitia of the larger cerebral arteries and veins The functional significance of their existence has however, not yet been clarified Falck *et al* also found that following bilateral cervical sympathectomy in rabbits the fluorescence in all cerebral vessels was abolished James *et al* (1969) performed acute unilateral cervical sympathectomy and confirmed that the resting rCBF was then increased (Holmqvist *et al* 1957) Compared to controls in the high pressure range a disappearance of autoregulation was further reported following the unilateral sympathectomy Their results indicate that the adrenergic innervation of the brain vessels would be of importance for the autoregulation of rCBF

However with a very similar experimental setup Chikamitsu *et al* (1968) and Waltz *et al* (1970) reported the same findings as in the present series *i e* persistence of autoregulation One reason for the discrepancy between the study of James *et al* (1969) and the present one, could be that Purves *et al* carried out an acute unilateral sympathectomy To obtain a complete sympathetic denervation of the cerebral vessels we therefore performed bilater[illegible] sympathect[illegible] since Nielsen and Owman (1967) have shown that a unilatera[illegible] pathectomy [illegible] just reduce the fluorescent adrenergic nerve fibers Furtherm[illegible] and Euler (1963) demonstrated that 8 hrs after sympathectomy of [illegible] nd in the rat the amount of noradrenaline was r[illegible] cared completely in 24 hrs

Since there might be some species differences, Owman (1970, personal communication) suggested to wait about 2 weeks after sympathectomy before experimentation in order to achieve a total loss of noradrenaline in the walls of the cerebral vessels Langer *et al* (1967) has pointed out that supersensitivity of the receptor following sympathetic dennervation increases very rapidly after 48 hrs to reach 800 times the initial sensitivity after 1 month However, this applies to receptors in the nictitating membrane and it cannot be presumed that the receptors in the cerebral arteries react in a similar manner In fact, direct effects of noradrenaline upon the smaller cerebral arteries have not been possible to demonstrate in micro injection experiments in vivo (Wahl *et al* 1970)

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From the Department of Physiology, University of Goteborg Sweden

Studies on the Intestinal Vasodilatation Observed after Mechanical Stimulation of the Mucosa of the Gut

By

BJORN BIBER, OVE LUNDGREN and JOAR SVANVIK

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Abstract

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augmentation was completely lidocaine, a local anesthetic,

Intake of food leads to a moderate increase of splanchnic blood flow as has been demonstrated in both man and animal (Brodie, Cullis and Halliburton 1910 Brodie and Vogt 1910, Herrick *et al* 1934 Lowenthal Harpuder and Blatt 1952, Brandt *et al* 1955 Reininger and Sapirstein 1957, Grim and Lindseth 1958 Bursadoun and Reid 1962, Burns and Schenk 1967, Varro *et al* 1967 Fronek and Stahlgren 1968, Vatner, Franklin and van Citters 1970) This blood flow increase has been regarded as one component in the physiological response pattern evoked during digestion, and has therefore been characterized as a functional hyperemia When, however, it comes to the underlying mechanism responsible for this blood flow increase no explanations have been presented In a preliminary paper (Biber *et al* 1969) it was reported that mechanical stimulation of the jejunal mucosa can induce an augmentation of total intestinal blood flow in cats This intestinal vasodilatation may possibly be partly responsible for the functional hyperemia seen during digestive work

The aim of the present investigation was to further study the blood flow response evoked by such a local intestinal mechanical stimulus. In addition, the intestinal vasodilatation was investigated with a vital microscopy technique. Thus, below are reported experiments in which the response was studied after different types of mechanical stimulation, after acute intestinal denervation, during sympathetic vasoconstriction and during the influence of pharmacological agents blocking either nerve conductivity or pharmacological receptors in autonomic ganglia and vascular effector cells.

Methods

A. *Operative procedures*

Experiments were performed on 23 cats of both sexes with body weights varying between 2.0 and 4.5 kg. The animals were deprived of food for at least 24 hrs and were after an initial anesthesia with ether anesthetized with chloralose i.v. (50—70 mg/kg b.w.). The body temperature was kept at 37° to 38° C by means of a heating pad under the animal.

The abdomen was opened in the midline after insertion of a tracheal cannula and a jejunal segment immediately below flexura duodeno-jejunalis was chosen for the experiment. The length of the segment was about 20 cm and the total weight including the mesenteric lymph nodes amounted to 20—35 g. The remainder of the small intestine, the spleen, the great omentum and the colon were extirpated. The adrenal glands were in some experiments excluded from the blood circulation by ligatures. In other experiments one adrenal gland was denervated and the other excluded by ligatures.

After heparinizing the animal, the left femoral artery was cannulated and connected to a mercury manometer to record mean arterial blood pressure. After cannulation of the superior mesenteric vein the venous outflow from the jejunal segment was measured by an optical drop recorder unit operating an ordinate writer recording on smoked kymograph paper. The blood was returned to the animal *via* a funnel connected to a catheter in a jugular vein. During the experiments the jejunal segment was carefully wrapped in saline soaked gauze and kept at body temperature by means of a thermocouple recorder and an infra red lamp.

In most experiments all nerves in the tissues along the superior mesenteric artery close to the mesenteric root were cut, thereby accomplishing a postganglionic sympathetic and preganglionic parasympathetic denervation. In some experiments the nerves in the same region were carefully dissected and placed on ring electrodes and stimulated electrically by means of a Grass stimulator Model S 5.

In 3 expts skeletal muscle blood flow was measured simultaneously with the intestinal blood flow. The venous outflow of one acutely denervated leg was diverted through an optical drop recording unit by cannulating the femoral vein. The leg was skinned and the paw was excluded from the blood circulation by ligatures to minimize the admixture of skin blood flow in the femoral vein. Blood was returned to the animal *via* a catheter in the femoral vein of the opposite leg.

In 3 expts microscopic observations were made of the villous blood circulation. In these experiments the small intestine was cut open with thermocautery along the antimesenteric border and placed on a cork plate. A Zeiss dissection microscope (Model OPMI) with 40× magnification in direct illumination was used for the observations.

B. *Experimental procedures*

Mechanical stimulation of the mucosa was performed in four different ways. 1) Usually a polyvinylchloride (PVC) plastic tube (outer diameter 5 mm) was used. It was inserted into the lumen of the jejunal segment at its proximal end and slowly pushed while rotating it towards the distal end of the segement after which it was withdrawn in the same manner. 2) In some experiments mucosal stimulation was achieved by pulling carefully a 2 cm long piece of the PVC plastic tube through the jejunal lumen from the proximal to the distal end and back again by means of a soft string. When mechanical stimulation of the jejunal mucosa was performed as described under 1) and 2) the PVC tube was in contact with the mucosa for 20—30 sec. 3) In some experiments mechanical stimulation was accomplished by means of a rapid flushing of 50 ml saline at body temperature through the jejunal lumen. 4) In the vital microscopy experiments the mucosa was softly stroken with a glass rod or the end of a pair of forceps.

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Results

A *General characteristics of the intestinal vasodilatation evoked by mechanical stimulation of the jejunal mucosa*

Mechanical stimulation of the jejunal mucosa with a PVC tube was performed in 20 cats In all experiments this produced a jejunal blood flow increase, the magni[illegible] tude of which ranged from 30 to 140 per cent of the control blood flow The flow response was evident 20—30 sec after the start of the mechanical stimulation and reached its maximum within approximately 30 sec (Fig 1—8) The time for the return of blood flow from maximum to control level varied between 2 and 15 min Fig 1 illustrates 2 typical experiments where panel B shows a pattern of transient blood flow increase and panel A illustrates a more slowly passing vasodilatation

Repetetive mechanical stimulations of the mucosa could be performed in the course of one experiment without diminishing the induced blood flow increase In fact, blood flow could be increased to the same level by two mechanical [illegible] tions performed within a 3 min period as illustrated in Fig 2 No [illegible] changes in systemic blood pressure were observed during the blood flow increase produced by the mechanical stimulation of the mucosa No [illegible] observed in connection with these mechanical stimulations

In Fig 2 a transient blood flow decrease can be observed just [illegible] the mechanical stimulation This flow decrease was probably caused by a [illegible] stasis secondary to a slight pulling of the mesentery of the jejunal [illegible]

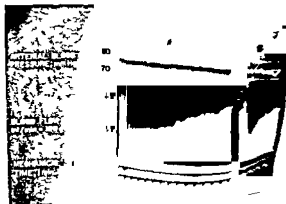


Fig 1 *Panel A* Cat 3 7 kg The effect of a mechanical stimulation of the jejunal mucosa on total venous outflow from gut Note the slow return to the control blood flow level
Panel B Cat 3 0 kg An experiment similar to that of panel A Note the comparatively short intestinal blood flow augmentation

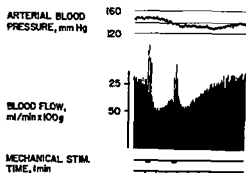


Fig 2 Cat 3 5 kg The effect of two consecutive mechanical stimulations of the jejunal mucosa on total intestinal blood flow It can be seen that blood flow is increased to the same flow level after the two stimulations

inevitably, but to different degrees, occurred in the beginning of each mechanical stimulation period when the jejunal segment was lifted and the plastic tube was introduced into the jejunal lumen This effect can thus be regarded as a 'stimulation artefact

In 3 expts the jejunal segment was cut open longitudinally at the anti mesenteric border for direct visual observation and the mucosa was lightly stroken with a glass rod or the tip of a pair of forceps from the proximal to the distal end of the segment A mucosal blanching was observed for about 5 sec immediately upon stimulation located along the line of mechanical stimulation A gradually spreadening red ness of the mucosa from the line of stimulation was then seen The maximal width of the mucosal flushing was about 5 mm on each side along the line of stroke and the redness could be observed for about 30 sec A small increase in total blood flow occurred concomitantly with the mucosal flushing When the intestinal segment was viewed under a microscope with direct illumination at 40 × magnification the macroscopic colour changes were revealed to be caused by a changed villous blood content indicating a villous vasoconstriction followed by a vasodilatation No villous movements were microscopically observed as a result of the mechanical stimulations

B *Different methods for producing the intestinal blood flow increase*

Insertion of a PVC plastic tube in the jejunal lumen was used as the standard method of provoking the blood flow increases of the present study However, some other techniques of stimulating the jejunal mucosa mechanically were also tested for their ability to induce an intestinal blood flow augmentation Fast flushings of 50 ml body-warm saline through the jejunal lumen in 3 expts resulted in a 15—50 per cent blood flow increase This vasodilator response was thus smaller but in other aspects similar to that obtained by PVC plastic tube stimulation In the experiments in which the mucosa was observed under a microscope a light strokening with a glass rod or the end of a pair of forceps induced a small but obvious blood flow

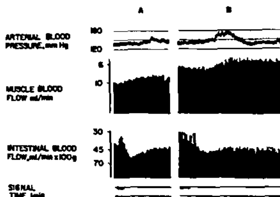


Fig 3 Cat 2 7 kg
Panel A The effect of mechanical stimulation of the jejunal mucosa (signal) on intestinal blood flow (lower flow registration) and skeletal muscle blood flow (upper flow registration) Note the abscence of any effect on skeletal muscle blood flow in the face of a transient augmentation of intestial blood flow
Panel B The effect of bile introduced into the gut lumen (signal) on intestinal blood flow (lower flow registration) and on skeletal muscle blood flow (upper flow registration) Note the prolonged increase of intestinal blood flow and the absence of any effect on skeletal muscle blood flow

increase Pulling of the jejunal mesentery without interfering with the venous out flow had no noticable effect on total intestinal blood flow

In other experiments the slow introduction of 2—3 ml body warm bile in the jejunal lumen induced a prolonged intestinal vasodilatation as is illustrated in Fig 3 B (lower right panel) No blood flow change was seen after introducing the same amount of saline Glucose solutions (0 75 M) and fat solutions (corn oil) seemed to be ineffective in producing any blood flow response, when applied in the jejunal lumen in amounts not causing distension

When the above mentioned type of jejunal vasodilatation was produced by either mechanical stimulation of the mucosa or by introduction of bile no vasodilatation was ever recorded in a skeletal muscle preparation as indicated by the results of three experiments If anything a small decrease in muscle blood flow occurred (upper panels of Fig 3)

C *Studies on the intestinal vasodilatation*

1 Influence of sympathetic nervous vasoconstriction In three experiments the effect of mechanical stimulation of the mucosa on jejunal blood flow was studied during sympathetic nervous vasoconstriction Electrical stimulation of the regional sympathetic fibres led to an immediate drastic decrease of intestinal blood flow, which however in the course of some minutes again increased and within 2—4 min reached an intermediate blood flow level (Folkow *et al* 1964)

The mechanical stimulation procedure induced an intestinal blood flow increase of the usual magnitude, in spite of the concomitant sympathetic vasoconstriction This proved to be the case both during the initial drastic flow decrease and during the 'steady state' phase of neurogenic vasoconstriction The latter observation is illustrated in Fig 4 It is evident from Fig 4, that the mechanically induced blood flow increase was even greater than the blood flow augmentation recorded during the hyperemia that occurred upon cessation of constrictor fibre stimulation

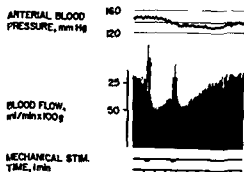


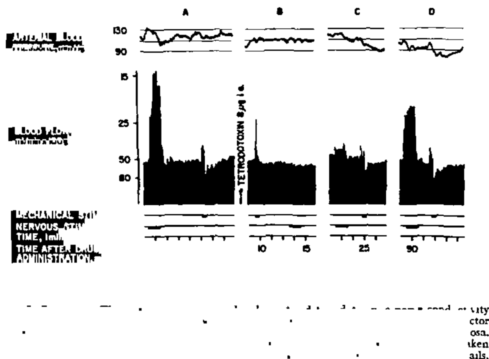
Fig 2 Cat 3 5 kg The effect of two consecutive mechanical stimulations of the jejunal mucosa on total intestinal blood flow It can be seen that blood flow is increased to the same flow level after the two stimulations

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the other hand, still possible to induce a pronounced vasodilatation by a close i a injection of isopropylnoradrenalne (not shown in the Fig), indicating that vascular tone and smooth muscle reactivity was good About 20 min after the drug administration, there could be observed a small blood flow increase upon mechanical stimulation and a small blood flow decrease occurred upon direct sympathetic stimulation, indicating a returning nerve conductivity (Fig 5 C) After about 90 min the blood flow reactions to mechanical mucosal stimulation and to sympathetic stimulation had largely returned to those seen during control conditions (Fig 5 D)

4 *Effect of a local anesthetic agent* Four experiments were performed in which the nerve blocking properties of a local anesthetic agent, lidocaine were utilized The solutions were slowly given into the jejunal lumen in volumes not causing distention, and left in contact with the mucosa for about 5 min This procedure proved to abolish or drastically decrease the vasodilator response seen upon mechanical mucosal stimulation To test that no unspecific irreversible damage had been caused to the jejunal tissue by the drug application the drug was washed away from the mucosa by flushing body warm saline through the jejunal lumen After this procedure the jejunal segment regained its ability to react with vasodilatation after

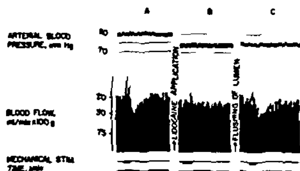


Fig 6 Cat 3 5 kg The effect of intraluminal application of lidocaine a local anesthetic agent, on the intestinal blood flow increase observed after mechanical stimulation of the jejunal mucosa. It can be seen that lidocaine totally abolishes the mechanically evoked blood flow response (panel B) After flushing the intestinal lumen with a physiological saline solution the intestinal blood flow increase again appears (panel C)

mechanical mucosal stimulation thus indicating that the drug effect was reversible and not caused by any unspecific cell damage A typical experiments is illustrated in Fig 6 which shows jejunal reactions prior to (A), during (B) and after (C) the administration of lidocaine

5 Effect of pharmacological receptor and ganglionic blocking agents Atropine (1 mg/kg b w) was administered i v in 16 expts The vasodilatation after mechanical stimulation was however still observed (Fig 7 A) Furthermore, the i v consecutive administration of 2 separate β receptor blocking agents propranolol (1 mg/kg b w) and alprenolol (1 mg/kg b w) in 2 expts did not abolish the vasodilatation ponse as is illustrated in Fig 7 B Thus the mechanically induced vasodilatation was not mediated *via* cholinergic or β receptor stimulating transmittors

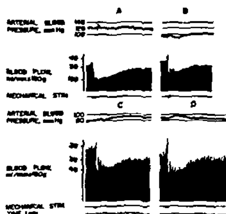


Fig 7 Cat 3 8 kg The effect of various pharmacological blocking agents on the blood flow response induced by mechanical stimulation of the jejunal mucosa. The experiment of panel A was performed on an atropinized animal (1 mg/kg b w Between panels A and B two β receptor blocking agents (propanolol 1 mg/kg b w and alprenolol 1 mg/kg b w) were given i v The α receptors were blocked by means of phentolamine (1 mg/kg b w) and phenoxybenzamine (2 mg/kg b w) administered i v between panels B and C Finally between panels C and D a cholinergic ganglionic blocking agent chlorisondamine was given i v (4 mg/kg b w) It is evident that none of the administrated drugs were able to abolish the intestinal blood flow response evoked by the mechanical stimulation

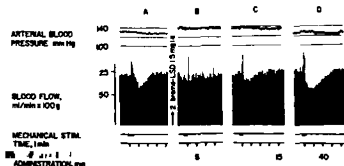


Fig 8 ...
to block ...
increase ...
and D ...
seen on mechanical stimulation of the jejunal mucosa

The possibility remained, however, that the vasodilatation was caused by a diminished tonic activity in local nervous fibre arrangements that exert their action by means of α receptor stimulating transmittors To rule out this possibility two separate α receptor blocking agents, phentolamine (1 mg/kg b w) and phenoxybenzamine (2 mg/kg b w) were given in 6 expts The effectiveness of the α receptor blockade was proven by the total abolishment of vasoconstriction upon direct vasoconstrictor fibre stimulation Nevertheless, vasodilatation was induced after mechanical stimulation, as is illustrated in Fig 7 C

Finally, in order to investigate whether cholinergic ganglionic synapses are involved in a possible local vasodilator reflex arc, a ganglionic blocking agent was used in four experiments Chlorisondamine, which is considered to block acetylcholine mediated transmission in ganglionic synapses in both the sympathetic and the parasympathetic nervous systems (Plummer *et al* 1955) was given i v (2—4 mg/kg b w) A marked blood pressure drop (40—60 mm Hg) occurred after the drug administration presumably because of a general blockade of sympathetic vasoconstriction This blood pressure fall could partly be compensated by i v infusion of a Dextrane Tyrode solution and by elevation of all the extremities of the animal However the jejunal vasodilatation response upon mucosal mechanical stimulation could still be elicited, as is illustrated in Fig 7 D

6 Effect of 5 hydroxytryptamine receptor blocking agent In 3 expts 2 bromolysergic acid diethylamide (2 bromo-LSD) a drug that is considered to have 5 hydroxytryptamine (5 HT) receptor blocking properties (Gyermick 1966) was

used The drug was administered i.a to the jejunal segment by an injection technique similar to that described above for tetrodotoxin The results are illustrated in Fig 8 As is evident from this figure a complete abolishment of the intestinal blood flow response to mechanical stimulation was caused by the administration of 2 bromo-LSD (compare panels A and B of Fig 8) The vascular response to mechanical mucosal stimulation returns slowly after the drug administration (Fig 8 C and D), probably reflecting the slowly fading effect of the 5-HT blocking agent Vascular smooth muscle reactivity to isopropylnoradrenaline persisted throughout these experiments to judge by the vasodilatations observed on intra arterial injections of the drug

Discussion

It was demonstrated in the present study that even weak mechanical stimulation of the jejunal mucosa induces a transient increase of total jejunal blood flow amounting to 30 140 per cent of control The jejunal vasodilatation was, at least partly, localized to the mucosal layer, since a villous vasodilatation was observed upon mechanical stimulation when viewing the mucosa under a dissection microscope It was also evident from the vital microscopy studies that villous movement or intestinal peristalsis did not accompany the mechanically induced jejunal blood flow increases.

In order to explore whether the mucosal mechanical stimulation released a vasodilating agent into the intestinal venous blood which may secondarily relax vascular smooth muscles also in other systemic vascular beds blood flow in the hind limb skeletal muscle was recorded in some experiments concomitant with the intestinal venous outflow Since the jejunal venous blood in the experimental set up of the present study did not pass directly through the liver but via the jugular vein to the heart a vasodilating effect on other vascular beds might be observed if the concentration of the dilating agent in the intestinal venous blood was high enough. No skeletal muscle vasodilatation was however seen upon mechanical stimulation of the jejunal mucosa, indicating that in case a vasodilator agent was released locally and entered the venous blood it did so in only very small amounts and/or it was rapidly inactivated in the blood

Spinal reflexes affecting motility and blood flow are known to exist in the cat. Thus the distention of one intestinal segment produces a reflex inhibition of intestinal motility and an intestinal vasoconstriction (Johansson and Langston 1964 Furthermore autonomic nervous reflexes with synapses in peripheral ganglia have been described in the splanchnic region (cf Job and Lundberg 1952) In order to test whether the vasodilatations of the present study were induced via similar nervous reflexes experiments were performed on intestinal segments that were sympathetically and parasympathetically denervated This was in most cases accomplished by carefully dissecting and cutting all nerves around the superior mesenteric artery and vein distally to the sympathetic ganglia Further in three experiments the splanchnic nerves, containing the preganglionic sympathetic fibres as well as the vagi were cut

on both sides during the course of the experiment No impairment of the usual intestinal vasodilatation was seen in any of these experiments suggesting that the intestinal vasodilatation observed in the present study was not relayed *via* spinal or sympathetic ganglionic structures

While thus acute pre or postganglionic denervation of autonomic fibres proved to be without effect on the mechanically induced jejunal vasodilatation, there still existed the possibility that strictly intramural nervous mechanisms, not affected by the "extrinsic" denervation, could be involved Such nervous structures cannot be mechanically interfered with in an *in vivo* preparation of the type used in the present study, but can be influenced by *e g* bloodborne nerve blocking agents A suitable agent for this purpose is the fish poison tetrodotoxin which selectively blocks nerve conductivity without interfering with smooth muscle activity *per se* (Gershon 1967) The i a administration of this drug caused a transient abolishment of the mechanically induced vasodilatation as well as of the intestinal vasoconstriction evoked by regional sympathetic stimulation, while vascular reactivity to bloodborne vasodilator agents remained largely unchanged Thus these experiments gave reason to believe that a *local* nervous mechanism was involved and essential for the jejunal blood flow increase observed upon mechanical stimulation of the intestinal mucosa This conclusion was further strengthened by the experiments in which it was demonstrated that lidocaine, a local anesthetic agent, introduced into the intestinal lumen abolished the mechanically induced intestinal vasodilatation The latter experiments furthermore suggest that the nervous structures involved are, at least partly, located close to the mucosal surface

In one series of experiments different receptor and ganglionic blocking agents were tested as regards their effects on the intestinal vasodilator response It was clearly shown that neither cholinergic nor adrenergic blocking agents had any noticable effect on the mechanically evoked blood flow increase Further no effect was observed after administration of a cholinergic ganglionic blocking agent

The intestinal tissue contains large amounts of 5 hydroxytryptamine (5 HT) mainly located in the enterochromaffin cells (Vialli 1966 Penttila and Lempinen 1968) but also to some extent in some of the nerve cells that constitute the intra mural plexa (Gershon and Ross 1966) The physiological function of these 5 HT stores is largely unknown although theories associating 5 HT with bowel motility particularly peristalsis have been presented (Haverback and Davidson 1958 Bulbring 1961 Kottegoda 1969) Since the central nervous system evidently contains neurons utilizing 5 HT as a transmittor it seemed justified to test whether the jejunal vasodilatator mechanism studied might be conveyed *via* nerve endings releasing 5 HT 2 bromo LSD was used in these experiments and was shown to transiently abolish the mechanically induced intestinal vasodilatation without effecting vasodilatations caused *e g* by injected vasodilator agents

To summarize the experiments of the present study have clearly demonstrated that mechanical stimulation of the jejunal mucosa induces a substantial, transient intestinal vasodilatation, at least partly localized to the villi Furthermore they seem

to suggest the involvement of an intramural nervous mechanism that in some way or other is dependent of 5-HT release which would in turn, be responsible for the mechanically evoked intestinal vasodilatation

It is not possible from the experiments of the present study to draw any conclusions concerning the anatomical arrangement of the nervous structures involved in the intestinal vasodilatations studied *A priori* one of two arrangements seems probable The arc may be arranged in a manner resembling the peristaltic reflex (Kottegoda 1969) *i e* an intramural reflex arc with one or several synapses In such a nervous reflex arc 5-HT could exert its action on the mucosal receptor, at a synapse or at the neuromuscular junction An axon reflex similar to that described for the skin (Folkow, Strom and Uvnas 1950) seems to be the other possible arrangement 5 HT could in such a reflex arc act on the receptor or at the neuromuscular junction.

In some experiments it was noted that a long standing jejunal blood flow increase was induced by administration of bile into the lumen This observation was not further analyzed in the present study Consequently, one cannot exclude the possibility that this type of jejunal vasodilatation is produced by a different mechanism than the vasodilatation observed upon mechanical stimulation It has been demonstrated that administration of isotonic glucose and glycine solutions into the lumen of isolated jejunal loop of the dog induce a local blood flow increase (Varro *et al* 1967) Furthermore Fara Rubinstein and Sonnenschein (1969, 1970) reported that blood flow in the superior mesenteric artery increased after instillation of milk or corn oil in the duodenum reflecting a vasodilatation in the intestine and/or in the pancreas These observations indicate the existence of mucosal receptors which are sitive to a variety of chemical and mechanical stimuli and which may affect testinal blood flow Further studies concerning the influence of different types of mucosal stimuli on the intestinal blood flow are planned in order to elucidate this matter

As pointed out in the introduction the mechanism underlying the functional hyperemia of the gut is not known It can however, be ruled out that extrinsic nervous influence plays any role since earlier investigations have failed to demonstrate the existence of any centrally controlled vasodilator fibres supplying the vessels of the small intestine (Kewenter 1965) From a theoretical point of view it also appears more plausible that the functional hyperemia of the gut is controlled by local rather than remote factors since extrinsic nerves or blood borne hormones would produce a diffuse vasodilatation of the whole gastro-intestinal tract. To what extent the mechanically induced vasodilator response of the present study is of any importance in explaining the functional hyperemia of the gut is not known at present. The mechanical stimulus needed to evoke the intestinal vasodilatation seems however to be comparatively small It is therefore possible that such stimuli may be induced by the normal small intestinal motility Further investigations along these lines are intended

The excellent technical assistance of Mrs Dagny Johansson and Miss Lisbeth Larsson is gratefully acknowledged

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Acta physiol. scand 1971. 82 191—201

From the Department of Physiology, University of Goteborg, and Research Laboratories, AB Leo, Halsingborg Sweden

Rapid Interaction between Gonadotrophic Hormones and the Prepubertal Rat Ovary

By

KURT AHREN, LARS HAMBERGER and TORSTEN PERKLEV

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Abstract

AHREN, K., L. HAMBERGER and T. PERKLEV *Rapid interaction between gonadotrophic hormones and the prepubertal rat ovary* Acta physiol scand 1971 82 191—201

Lactic acid production by isolated ovaries from prepubertal rats was used as an experimental system to characterize the initial interaction between luteinizing hormone (LH) and human chorionic gonadotrophin (HCG) on the one hand and the ovarian tissue on the other hand [illegible]

in hormone free medium These effects did not become more pronounced when the hormone was also present during the entire 2 hr incubation period when lactic acid production was [illegible]

The precise nature of the initial interaction between a hormone and its specific target tissue is not yet known for any hormone It is however usually assumed that the very first step in a hormonal interaction with a target cell is a binding of the hormone to some cellular constituent — a binding site or a receptor site' Evidence supporting such a concept has been obtained during recent years particularly in the field of steroid hormone research It has for instance been shown that following both *in vivo* and *in vitro* administration of oestrogens the steroid hormone is selectively accumulated by target organs such as the uterus and vagina (*e g* Jensen and Jacobsson 1962 Terenius 1968) Isolation and partial purification of oestrogen

binding macromolecules from rat and rabbit uterus have, in addition been reported (for ref see Gorski *et al* 1968)

For the polypeptide hormones the first experiments designed to study the problem of hormone binding to the target tissue were a series of experiments with insulin (Stadie *et al* 1949) These investigators found that the rat diaphragm incubated briefly in a medium containing insulin and then washed thoroughly in insulin free medium showed a persistent effect of insulin on glycogen synthesis Experiments have later been reported indicating binding of growth hormone to muscle tissue (Kostyo and Schmidt 1961), of adrenocorticotrophic hormone (ACTH) to rat adrenals (Birmingham and Kurlents 1958), and of thyroid stimulating hormone (TSH) to thyroid tissue (Pastan Roth and Macchia 1966) In the last mentioned study it was found that dog thyroid slices exposed to TSH and then thoroughly washed in hormone free medium showed a persistent hormone effect When the thyroid tissue was exposed to an anti TSH serum after the initial interaction between the hormone and the tissue the hormone effect was abolished Pastan and co-workers concluded from their study that the persistent hormone effect was due to the continued presence of relatively intact hormone at a readily accessible tissue site , and that the locus of the initial binding of TSH to the cell is not inside the cell but at the superficial surface of the cell

The present experiments were undertaken in order to analyse the initial interaction between gonadotrophins and the prepubertal rat ovary It is well established that gonadotrophic hormones have a pronounced stimulatory effect on the *in vitro* glycolysis of such ovaries and that this effect is obtained both when the hormones are added *in vitro* to the isolated ovaries and when they are injected to the rats before removal of the ovaries (for ref see Ahrén Hamberger and Rubinstein 1969) In the present study the rate of *in vitro* glycolysis of prepubertal rat ovaries was studied after brief *in vivo* or *in vitro* exposure to bovine luteinizing hormone (LH) or human chorionic gonadotrophin (HCG) The influence of an anti HCG serum added before together with or after the addition of the hormone to the incubation medium has also been studied

Methods

Animals

Female rats 24—26 days old of the Sprague Dawley strain were used The rats were given a semisynthetic diet (Gustafsson 1959) and water *ad libitum* but were deprived of food 24—26 hr before dissection

Hormones

[illegible] Section 87 [illegible]

71 75 16) was a highly purified material with a specific activity of 13 000 IU/mg [illegible] assayed against the Second International Standard Preparation of Human Chorionic Gonadotrophin

LH was dissolved in 0 9 % NaCl (200 μg/ml) for the injections, control rats were injected with 0 9 % NaCl LH and HCG were dissolved directly in Krebs bicarbonate buffer for the *in vitro* experiments

Anti HCG serum

The antiserum was prepared by immunizing rabbits according to the technique described by Wide (1962) A highly purified HCG preparation (11 700 IU/mg) in Freund s adjuvant was

HCG which completely inactivated the antiserum was evaluated by the haemagglutination inhibitior reaction It was then found that roughly 125 μg of our HCG preparation was required to eliminate the effect of 1 ml ARS HCG 21

Removal and incubation of ovaries

The rats were killed by cervical fracture The ovaries were rapidly removed and trimmed of bursae and extraneous tissue under a dissection microscope as described in previous papers

wet weight

In another series of experiments the ovareis were exposed to hormone and/or anti HCG serum *in vitro* during a preincubation period before being transferred to the final incubation flasks where lactic acid production was determined after a 2 hr incubation period as after the *in vivo* administration of the hormone

Measurement of lactic acid production

Lactic acid produ riod was calculated from the amount cubation period for reasons discussed s determined by an enzymatic method Mohme Lundholm and Svedmyr 1963 Lundholm Mohme Lundholm and Vamos 1963)

Statistical procedure

Mean values are given ± standard error of the mean Comparison between different groups was performed according to Student s t test A p value of 0 05 or less was considered significant in this study

Results

In vivo administration of LH

In previous experiments where ovarian glycolysis *in vitro* has been studied after *in vivo* administration of LH the hormone has always been injected to the rats 30—240 min before extirpation of the ovaries The purpose of the present series of experiments was to investigate whether LH could stimulate the *in vitro* glycolysis of prepubertal rat ovaries when LH was injected *in vivo* just a few minutes before extirpation of the ovaries

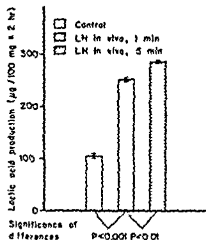


Fig 1

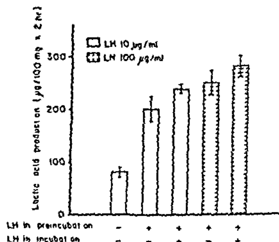


Fig 2

Fig 1 Lactic acid production by isolated ovaries from prepubertal rats injected with bovine LH i v 1 or 5 min before extirpation of the ovaries — Lactic acid production was measured during a 2 hr incubation period at +37° C in Krebs bicarbonate buffer containing 5 5 mM glucose — Each value represents the mean of 3 observations and standard errors are indicated by vertical lines

Fig 2 Lactic acid production by prepubertal rat ovaries preincubated or continuously incubated with bovine LH (10 and 100 µg/ml medium) — Lactic acid production was measured during a 2 hr incubation period at +37° C in Krebs bicarbonate buffer containing 5 5 mM glucose with and without LH The ovaries were first taken through the following 3 preincubation periods (at +1° C) a) wash for 1 min in buffer b) preincubation for 1 min with LH and c) wash for 5 min in buffer — Each value represents the mean of 4 observations and standard errors are indicated by vertical lines

TABLE I Lactic acid production by isolated ovaries from control rats and rats injected with bovine LH

Time between injection and extirpation of ovaries	Lactic acid production* (µg/100 mg 2 hr)			
Controls 5 and 30 min*	106	5	(16)	p < 0 001
LH 5 min	302	13	(12)	
LH 30 min	323	11	(8)	N S

... bicar 5 and NaCl tively 10 min

control groups

* Mean ± S E Number of observations in parenthesis N S — not significant

The rats were anesthetized with nembutal (4 mg/100 g b w) 20—30 min before the injection of LH The hormone was given in one intravenous (i v) injection (100 µg/100 g b w), 30 5 and 1 min respectively, before extirpation of the ovaries and ovarian lactic acid production was then measured during a 2 hr incubation period

TABLE II Lactic acid production by isolated ovaries from prepubertal rats during a 2 hr incubation period after preincubation with bovine LH or HCG[1]

Preincubation periods 1 min wash + {1 min LH or HCG} + 1 min wash		Lactic acid production[2] (µg/100 mg × 2 hr)	Significance of hormone effect
A	4° + 37° C		
	Controls	123 ± 16 (3)	
	LH (100 µg ml)	249 ± 9 (3)	$p < 0.005$
B	4° + 4° C		
	Controls	126 ± 11 (3)	
	LH (100 µg/ml)	241 ± 7 (3)	$p < 0.001$
C	4° + 4° C		
	Controls	125 ± 5 (4)	
	LH (10 µg/ml)	203 ± 10 (4)	$p < 0.001$
	HCG (5 µg ml)	185 ± 8 (4)	$p < 0.001$

[1] Lactic acid production was measured during a 2 hr incubation period at +37° C in Krebs bicarbonate buffer containing 5 5 mM glucose The ovaries were first preincubated for 1 min with LH or HCG The conditions of the preincubation periods are described in detail in the text
[2] Mean ± S E Number of observations in parenthesis

The results are given in Fig 1 and Table I It can be seen from Fig 1 that ovaries from rats injected with LH for only 1 min before extirpation of the ovaries produced more than 100 % more lactic acid during the following incubation period than simultaneously incubated ovaries from control rats The ovaries from the 5 min group had slightly higher ($p < 0.01$) lactic acid production

Table I shows experiments where ovaries from rats injected 5 and 30 min respectively, before the extirpation of the ovaries were compared It can be seen that lactic acid production was not significantly higher in the 30 min group than in the 5 min group

It was thus clear from these experiments that the prepubertal rat ovary had a markedly increased rate of glycolysis *in vitro* after as short a time as 1 min *in vivo* exposure to LH The effect was slightly more pronounced after 5 min *in vivo* exposure but an increase in exposure time to 30 min did not further increase the [illegible] It was therefore decided to investigate whether prepubertal rat ovaries [illegible] LH or HCG *in vitro* i e preincubated with the hormone for 1—5 min also [illegible] persistent hormone effect when incubated again after careful washing [illegible] medium

In vitro administration of LH and HCG

In the first series of experiments summarized in Table II, the [illegible] through the following 3 preincubation periods before the final [illegible] acid production was measured a wash for 1 min in plain [illegible] incubation for 1 min in buffer (1 ml) containing the [illegible] 1 min in plain buffer (2 ml The preincubations were perf[illegible] periment A (Table II and at 1° C in experiments [illegible]

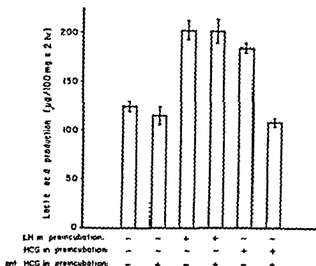


Fig 3 Influence of anti HCG on the effect of bovine LH and HCG when the antiserum was added together with the hormone — Lactic acid production by prepubertal rat ovaries was measured during a 2 hr incubation period at +37° C in Krebs bicarbonate buffer containing 5 5 mM glucose The ovaries were first taken through the following 3 preincubation periods (at +1° C) *a*) wash for 1 min buffer, *b*) preincubation for 1 min either with the hormone (LH 10 µg/ml HCG 5 µg/ml) or with anti HCG serum (50 µl added to 1 ml buffer) or with the hormone and anti HCG and *c*) wash for 5 min in buffer Each value represents the mean of 4 observations and standard errors are indicated by vertical lines

Table II that ovaries preincubated in a medium containing 100 µg LH/ml, showed a markedly increased rate of lactic acid production during the following incubation period compared to ovaries which were preincubated in a medium without LH It is also evident that LH had quantitatively the same effect when the preincubations were performed at +1° C as at +37° C When the concentration of LH in the preincubation medium was decreased to 10 µg/ml (experiment C Table II a significant effect was still recorded even if the effect was quantitatively slightly less pronounced than that observed when the LH concentration was 100 µg/ml The addition of HCG in a concentration of only 5 µg/ml medium during the preincubation was found to be sufficient for stimulating lactic acid production during the final incubation period (experiment C Table II)

Another experiment (Fig 2) was designed to investigate whether an addition of LH to the final incubation period could produce an additional effect to that obtained by addition of the hormone during the preincubation period The conditions for the preincubation periods were the same as those in experiments B and C (Table II) except that the third preincubation period (wash in hormone free buffer) was increased to 5 min and that LH was added to the final incubation medium in two of the groups studied It can be seen from Fig 2 that the addition of 10 and 100 µg LH/ml during the final 2 hr incubation period did not produce significantly higher lactic acid production than when the hormones were present only during the preincubation period even though the mean values were slightly higher

Effects of anti HCG serum

The first experiment of this series is summarized in Fig 3 The ovaries were taken through the same 3 preincubation periods as those described above all three at +1° C The medium of the second preincubation period contained either the hor

TABLE III Inability of anti HCG to neutralize the effect of HCG when the antiserum was added after the hormone[1]

Group	Additions to media during preincubation periods				Lactic acid production[2] (μg/100 mg × 2 hr)
	1 min +1° C	1 min +37° C	5 min +37° C	5 min +37° C	
A	—	—	—	—	135 ± 8 (4)
B	HCG	—	—	—	183 ± 12 (4)
C	—	—	anti HCG	—	126 ± 10 (3)
D	HCG	—	anti HCG	—	198 ± 9 (4)

mone (LH or HCG) alone or anti HCG serum alone, or the hormone *and* anti HCG serum together The media of this second preincubation period were kept for 30 min at +37° C with continuous shaking before the start of the experiment in order to assure enough time for an antigen antibody reaction It can be seen from Fig 3 that addition of anti HCG serum alone during the preincubation period did not significantly change lactic acid production by the ovaries during the following 2 hr incubation It is also evident that anti HCG serum added together with the hormones, completely abolished the effect of HCG but did not influence the effect of the bovine LH at all

In the last mentioned experiment the hormone and the anti HCG serum were kept together for 30 min before addition of the ovaries It was however, found in other experiments (not given in detail in this paper) that it was enough to keep the hormone and the antiserum together for 1 min before addition of the ovaries to completely prevent the HCG effect

The following experiment summarized in Table III was designed to investigate whether the addition of anti HCG serum *after* the initial interaction between HCG and the ovaries could abolish the effect of the hormone The following 4 preincubation periods were used a) 1 min at +1° C with or without HCG 5 μg/ml medium) b) 1 min wash in plain buffer at +37° C c) 5 min at +37° C with or without anti HCG serum (50 μg/ml medium) and d) 5 min wash at +37° C It can be seen from Table III that anti HCG serum under these conditions did not abolish the effect of HCG

The experiments performed up to this point indicated that the anti HCG serum could neutralize the effect of HCG on the prepubertal rat ovary when added together with the hormone before the addition of the ovaries but not when added after the initial interaction between hormone and ovary The same conclusion can be drawn from another experiment summarized in Table IV where the experimental conditions for the preincubation periods were slightly different from those described

TABLE IV Influence of anti HCG on the effect of HCG under various experimental conditions[1]

Group	Preincubation periods (+37° C)		Lactic acid production[2] (μg/100 mg×2 hr)
	I = 6 min	II = 5 min	
A	Buffer	Buffer	88±12 (4)
B	Anti HCG	Buffer	102±16 (4)
C	Buffer 5 min + HCG 1 min	Buffer	206±21 (4)
D	HCG 1 min + Anti HCG 5 min	Buffer	179± 9 (4)
E	Anti HCG 1 min + HCG 5 min	Buffer	99± 3 (4)

[1] Lactic acid production by isolated ovaries was measured during a 2 hr incubation period at +37° C in Krebs bicarbonate buffer containing 5 5 mM glucose The ovaries were first taken through 2 preincubation periods with the addition of HCG (5 μg/ml medium) and/or anti HCG serum (50 μl/ml medium) as indicated in the Table

[2] Mean ±S E Number of observations in parenthesis *Significance of differences* A—B A—E and C—D N S, A—C $p < 0\ 005$ A—D $p < 0\ 001$, B—C $p < 0\ 01$

earlier in this paper In this experiment (Table IV) the ovaries were first taken through a 6 min preincubation period (at +37° C) with or without addition of HCG and/or anti HCG serum All ovaries were then washed for 5 min in plain buffer (= preincubation period II, +37° C) Lactic acid production by the isolated ovaries was then measured during a subsequent 2 hr incubation period It can be seen from Table IV a) that anti HCG serum alone did not significantly influence the ovarian glycolysis b) that HCG when present during 1 min of the first pre incubation period markedly increased the lactate production during the final incuba tion period, and c) that addition of anti-HCG serum 1 min before the addition of the hormone completely prevented the HCG effect while the addition of anti HCG serum 1 min after the addition of HCG did not influence the effect of the hormone

Discussion

In all previous experiments concerning the *in vivo* effect of LH on the rate of *in vitro* glycolysis of the prepubertal rat ovary the hormone has been injected to the rats 30—240 min before the extirpation of the ovaries (*e g* Ahren and Kostyo 1963 Armstrong, Kilpatrick and Greep 1963 Ahrén, Hamberger and Rubinstein 1968) When Armstrong and Greep (1962) investigated the *in vitro* glycolysis of slices of luteinized ovaries after *in vivo* administration of LH they found that minimal although statistically insignificant effects were observed as early as 30 min after LH injection', and they reported quantitatively better effects when the hormone was given 60—120 min before the extirpation of the luteinized ovaries In the present study with prepubertal rat ovaries a very marked effect of LH on the *in vitro* glyco lysis was obtained when the hormone was injected to the rats 1—5 min before the

extirpation of the ovaries The effect was not more pronounced when the hormone was administered 30 min before the start of incubation

The persistent effect of LH and HCG on ovarian glycolysis demonstrated in the present study on prepubertal ovaries exposed for 1—5 min to the hormone *in vitro* is in principal very similar to that demonstrated in experiments with insulin and muscle tissue (Stadie *et al* 1949), and with TSH and dog thyroid slices (Pastan *et al* 1966) Birmingham and Kurlents (1958) reported a similar persistent effect of ACTH on rat adrenals, but these investigators did not try shorter exposure time to the hormone than 5 min.

There is, however, one striking difference between the results of the present experiments and the experiments with insulin and muscle tissue Stadie and co-workers found that the persistent effect of insulin during the final incubation period was dependent upon the temperature during the preincubation period when the tissue was exposed to the hormone The insulin was found to be three times more active when this temperature was +37° C than when it was +1° C In the present study the effect of LH was the same whether the temperature during the exposure to the hormone was +37° C or +1° C (see Table II) From the experiments of Pastan *et al* (1966) with TSH and dog thyroid slices, it is not possible to conclude whether or not the effect of TSH is temperature dependent since all the preincubations were performed at +1° C It is also impossible to tell from the experiments of Birmingham and Kurlents (1958) whether the persistent effect of ACTH on the adrenals after preincubation with the hormone is temperature dependent.

The first result of interest in the experiments with anti HCG serum in the present study is the fact that it was possible to demonstrate an anti HCG activity of the anti HCG serum in the *in vitro* biological system used It has been pointed out by Robyn Diczfalusy and Finney (1968) that there is a great need for precise and statistically valid bioassay procedures' for the estimation of the gonadotrophin neutralizing potency of antisera All available biological assay methods require *in vivo* administrations of hormone and sera, often for several days (*e g* Robyn and Diczfalusy 1968 a), and these methods are therefore time consuming and laborious The observations of the present study suggest the possible eventual usefulness of an experimental system with isolated prepubertal rat ovaries as an *in vitro* bioassay method for anti HCG and anti LH activities

The observation that the anti HCG serum did not neutralize the effect of bovine LH in the present experimental system (see Fig 3) confirms earlier observations with immunological and biological methods that there are no cross reactions between anti HCG serum and ovine or bovine LH (*e g* Robyn and Diczfalusy 1968 b) in contrast to the well known cross reaction between anti HCG serum and human LH (Wide 1962)

The most interesting result of the present experiments with anti HCG serum at least in relation to the questions brought up in the introduction is the observation that addition of anti HCG serum *after* the initial 1 min interaction between HCG and the ovaries did not abolish the persistent effect of the hormone This was shown

very clearly under two slightly different experimental conditions (Tables III and IV), and this result is in marked contrast to those reported by Pastan *et al* (1966) with TSH, anti TSH serum and thyroid slices They found that the persistent effect of TSH *after* a preincubation period with TSH and thyroid slices for 15 min at $+1°$ C was completely abolished after addition of TSH antibodies Pastan *et al* (1966) also reported some results of experiments with rat diaphragms insulin and anti insulin serum They found in these experiments that addition of anti insulin to diaphragms previously exposed to insulin for 1 min substantially reduced the persistent effect of insulin

Pastan *et al* (1966) draw from their own experiments with TSH and insulin and from those mentioned above by other investigators with growth hormone and ACTH several more general conclusions concerning the initial interaction between polypeptide hormones and target cells Their first conclusion was that a persistent hormone effect as that described *e g* in the present study after short exposure to LH or HCG must be due to either (a) persistence of hormone intact or altered in or on the tissue or (b) the initiation of secondary reactions whose subsequent course is independent of the presence of hormone In the case of TSH and dog thyroid slices the first of these two explanations with a relative intact hormone bound to a superficial cell surface seems probable For LH and HCG stimulating the carbohydrate metabolism of the prepubertal rat ovary the results of the present study make it difficult to choose between the two above mentioned alternatives The persistent hormone effect after short exposure to LH or HCG might be due to persistence of hormone even though the effect could not be obliterated with antibodies but the hormone must then be bound to the cell or in the cell in a position where it can no longer be reached by the antibodies Another alternative is that there is a binding to the target cells of an altered hormone molecule which can no longer be neutralized with the antibodies produced against the original molecule The possibility cannot however be excluded that the persistent hormone effect of LH or HCG after a short exposure to the hormone is due to a rapid initiation of secondary reactions in the ovarian cells most likely then in the cell membranes whose subsequent course is independent of the presence of the hormone The fact that the persistent effect was evoked by the hormone even at $+1°$ C makes this last mentioned possibility less likely Further experiments are however necessary to elucidate in detail the mechanism for the initial interaction between the gonadotrophins and the ovary

There is another aspect of the results of the present experiments which seems important to point out An increased plasma level of LH caused by an increased release of LH from the hypophysis can apparently produce an effect of considerable duration even if the plasma level of LH decreases again very rapidly due to inactivation or excretion of the hormone This fact must be taken into serious consideration when one discusses the relationship between changes or lack of changes in urinary excretion pattern and plasma levels of gonadotrophins on the one hand and the changes of ovarian function during various phases of the reproductive cycle on the other hand

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From the Department of Physiology I Karolinska Institutet Stockholm Sweden

Effect of Prolonged Physical Training on the Catecholamine Levels of the Heart and the Adrenals of the Rat

By

INGEGERD ÖSTMAN and NILS O SJOSTRAND

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Abstract

ÖSTMAN, I and N O SJOSTRAND *Effect of prolonged physical training on the catecholamine levels of the heart and the adrenals of the rat* Acta physiol scand 1971 *82* 202—208

The effect of prolonged physical training on the noradrenaline content of the heart and the catecholamine content of the adrenal glands was studied on rats The trained rats showed a moderate but highly significant cardiac hypertrophy (heart weight of controls 1 32±0 01, trained rats 1 41±0 01 heart ratio (g heart weight/100 g b w) of controls 0 29±0 003 trained rats 0 34±0 003) There was no difference in cardiac noradrenaline concentration between trained and untrained rats (controls 0 69±0 04 μg/g trained rats 0 73±0 03 μg/g) There was a highly significant increase in adrenaline content of the adrenal glands expressed as μg adrenaline/kg b w (Controls 93±5 μg/kg trained rats 122±5 μg/kg) It is *concluded that prolonged physical training does not lower the amounts of sympathetic trans* mitter of the heart It is further suggested that an increased adrenaline content in the adrenal glands might be a manifestation of an adaptation to the increased demands upon the cardiovascular system in rats subjected to prolonged physical training

Intense exercise causes an enlargement of the heart combined with chronic brady cardia in rats (Hatai 1915, *cf* Tipton 1965, Leon and Bloor 1968) In recent studies (De Schryver *et al* 1967, 1969) a pronounced decrease in the concentration and the total amount of cardiac catecholamines in trained rats has been reported, and it has been suggested that this decrease of sympathetic transmitter might in part explain the bradycardia of the athletic heart The effect of training upon the size of the heart seems to be dependent upon the intensity of the training program (Leon and Bloor 1968) In the studies in which a decrease in catecholamines of the hearts or trained animals were determined (De Schryver *et al* 1967, 1969) the training load was comparatively low In the present investigation a comparatively intense training program was used The size of the hearts of trained animals and untrained controls was studied and the noradrenaline content was determined

Since it is known that the adrenals release catecholamines during exercise (Can non and Britton 1926 Ashkar *et al* 1968) it also seemed to be of interest to compare the total catecholamine content of the adrenals of trained und untrained animals

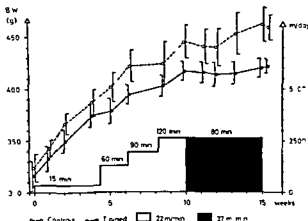


Fig 1 *Body weight increase during exercise* Empty circles and dotted lines controls (mean ± S D) Filled circles trained animals (mean ± S D) Training intensity indicated in the figure

Methods

24 male Sprague Dawley rats initially weighing about 300 g were used 11 of these rats were submitted to continuous training on a treadmill with an electrical fence at the far end of the apparatus The rest of the animals served as controls The following training procedure was used During the first 4 weeks of the experiment the rats were familiarized to the running

the rats were getting tired The number of contacts successively decreased over a period of 14 days, and the duration of the training period could therefore be gradually increased from 1 to 2 hrs in 4 weeks After 6 weeks the speed could be raised to 37 m/min with the animals still running At this high speed the animals had to run for 80 min/day (40 min + 40 min, with 10 min rest between the running periods) This type of heavy exercise was continued for 5 weeks The training program is shown graphically in Fig 1 After 4 days rest the animals were sacrificed by decapitation The hearts and the adrenals were taken out, cleaned and weighed The hearts were homogenized with an Ultra Turrax apparatus in 0 4 N perchloric acid Noradrenaline was extracted as previously described (Sjostrand and Swedin 1968) and determined fluorimetrically according to Chang (1964) In randomly chosen heart extracts from the controls and the trained animals adrenaline was determined with the same method using the conventional formula (Euler and Lishajko 1961) In no cases a significant amount of adrenaline was observed *i e* the adrenaline content constituted less than 10 % of the catecholamine content (*cf* Gunne 1963)

The adrenal glands were ground with chemically pure quarz sand in 2 ml of 0 4 N perchloric acid The adrenal extracts were oxidized and the concentration of noradrenaline and adrenaline was determined fluorimetrically according to Euler and Lishajko (1961) The catecholamines in hearts in adrenals were expressed as μg free base/g of wet tissue weight

Results

General observations

After a running period at the speed of 22 m/min the rats were almost unaffected and only a slight decrease in spontaneous activity and an increased water intake was observed during the first 30 min after exercise However, the speed of 37 m/min affected the animals markedly After a training period at this rate the rats salivated

TABLE I

Effect of prolonged physical training on body weight, body length and weight of the heart and the adrenal glands *

Groups	Initial body weight (g)	Final body weight (g)	Final body length (cm)	Heart weight (g)	Heart ratio (g/100 g)	Adrenal weight (mg)
Controls (n = 13)	324±4	459±3	27 2±0 2	1 32±0 01	0 29±0 003	42±2
Trained animals (n=11)	316±4	422±2 ***	27 0±0 1	1 41±0 01 ***	0 34±0 003 ***	5[illegible]±2 ***

n = number of animals
* Mean ± SEM
*** indicates difference from controls ($p < 0\ 001$)

profusely, and lay down in the cages only moving to drink copiously During the last week of the experiment the rats became less affected by the exercise and started apparently normal spontaneous activity about 15 min after exercise

Weight and catecholamine determinations

Fig 1 illustrates the increase in b w of the trained rats in comparison with the controls during the experiment The weight gains of the trained animals almost ceased during the period of intense training (37 m/min) Initial b w final b w, final body lengths heart weights absolute and related to body weight as heart ratio (g/100 g b w) and weights of the adrenal glands of the controls and the trained [illegible] are given in Table I As seen in the table there was no significant difference in initial b w of the two groups, but the final b w of the trained animals were significantly lower ($p < 0\ 001$) than those of the controls However the body lengths at the end of the experiment were almost identical There was a moderate but highly significant increase ($p < 0\ 001$) in heart weight of the trained rats The

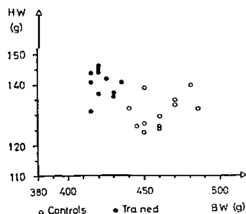


Fig 2 *Relation between heart weight and body weight* Symbols see figure

TABLE II Effects of prolonged physical training on the catecholamine contents of heart and adrenal glands Mean ± SEM

Groups	Total NA in heart (μg)	Concentration NA in heart (μg/g)	Total NA in adrenals (μg)	Total A in adrenals (μg)	% A of total CA
Controls (n 13)	0 90±0 05	0 69±0 04	7±1	43±2	85±2
Trained animals (n — 11)	1 03±0 04	0 73±0 03	9±2	51±2 **	86±2

n — number of animals
NA — noradrenaline
A — adrenaline
CA = catecholamines
** indicates difference from controls ($p < 0\ 01$)

effect of training on body and heart weights was still more evident from the heart ratios (g/100 g body weight) There was no overlap in heart ratio values of the two groups (Controls range 0 27—0 31, trained animals range 0 32—0 35) The correlation between heart weights and b w is shown in Fig 2 The weights of the adrenal glands were significantly increased in the trained animals ($p < 0\ 001$)

The catecholamine contents of the heart and the adrenals are given in Table II and Table III There was no significant difference between trained and untrained animals neither in total amount nor in the concentration of noradrenaline in the hearts The relation between the heart weight and the noradrenaline content is further illustrated in Fig 3

There was a significant increase ($p < 0\ 01$) in total adrenaline content, expressed as μg/pair of adrenals, in the adrenal glands of the trained animals When the adrenaline content was expressed as μg/kg b w the increase was highly significant ($p < 0\ 001$) There was, however, no significant difference of the adrenaline concentration, expressed as μg/g adrenal tissue, between the adrenal glands of trained and untrained animals The two groups show no overt difference in the noradrena

TABLE III Catecholamine content of the adrenals related to the weight of the adrenal glands and the body weight Mean ± SEM

Groups	NA μg/g adrenal tissue	A μg g adrenal tissue	NA μg kg body weight	A μg/kg body weight
Controls (n = 13)	177±23	1 035±70	16−2	93 5
Trained animals (n = 11)	160±27	940±53	21+4	122+5 ***

n = number of animals
NA — noradrenaline
A = adrenaline
*** indicates difference from controls ($p < 0\ 001$)

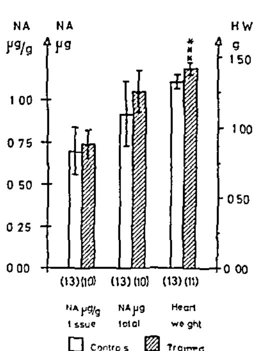


Fig 3

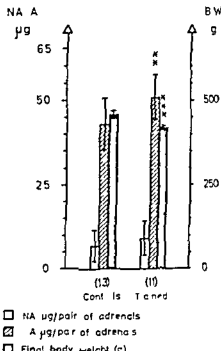


Fig 4

Fig 3 *Effect of training on the heart weights and noradrenaline contents in the heart* White columns controls (mean ± S D) Shaded columns trained rats (mean ± S D) *** indicates difference from controls ($p < 0\ 001$)

Fig 4 *Relation between catecholamine contents of the adrenals and the body weight* White columns noradrenaline content (mean ± S D), shaded columns adrenaline content (mean ± S D) and dark columns body weight (mean ± S D) ** indicates difference from controls ($p < 0\ 01$) *** indicates difference from controls ($p < 0\ 001$)

line content of the adrenals, neither in total amount nor relative to b w or adrenal weight The relation of the catecholamine contents of the adrenals and the b w in the controls and in the trained rats is illustrated in Fig 4

Discussion

It has been shown in the present study that physical training increases the weight of the heart and the adrenal glands The b w of the trained rats were decreased as compared with the controls but the body lengths were not These findings are in accordance with earlier results (Hatai 1915, and later authors) Neither the cardiac noradrenaline concentration nor the total amount of noradrenaline in the heart were decreased in the trained animals when compared with the controls The total amount of adrenaline in the adrenals from trained rats was increased in comparison with that of the controls while there is no significant difference in nor adrenaline content of the adrenals

Contradictory results have been attained by De Schryver *et al* (1967, 1969) who

found a highly significant decrease in heart catecholamine concentration of physically trained rats when compared with controls In their study there were no signs of cardiac hypertrophy, and the growth curves of trained and untrained animals were identical The different technique used for training the animals would seem to be the most likely cause of this discrepancy Leon and Bloor (1968) have found that an intermittent training as used by De Schryver *et al* (running 90 min at a speed of 750 m/hr = 12 5 m/min or at 500 m/hr = 8 3 m/min three times a week) is not heavy enough to induce cardiac hypertrophy Since the bradycardia in trained rats appears to be correlated with changes in b w, heart weight and heart ratio (Tipton 1965), it seems rather unlikely that De Schryver *et al* have achieved any degree of bradycardia in their trained animals Therefore their suggestion that a decrease in sympathetic transmitter substance might explain the bradycardia found in the athletic heart appears to lack experimental support

Cardiac hypertrophy induced by experimental aortic or pulmonary stenosis is followed by a decrease in cardiac noradrenaline concentration which is very marked in the severely hypertrophied or failing heart (Spann *et al* 1965 Krakoff *et al* 1968, Meerson 1969 Vogel *et al* 1969) Fischer *et al* (1965) showed that the cardiac noradrenaline concentration in the total amount of noradrenaline was observed only in severely hypertrophied hearts In this respect it is interesting to compare our results with those of Fischer *et al* (1965) With a moderate aortic stenosis resulting in a heart ratio of the same magnitude as that of our trained rats they observed a significant decrease in cardiac noradrenaline concentration while we find no tendency at all towards a decrease in noradrenaline concentration of the hearts

Thus the principle characteristics of moderate cardiac hypertrophy induced by experimental stenosis seems to be an increase in heart tissue without a corresponding increase in the amount of adrenergic transmitter, which results in a decrease in adrenergic transmitter concentration Our results suggest that the moderate hypertrophy induced by prolonged physical training is characterized by an increase in heart tissue without a decrease in adrenergic transmitter concentration

Since the early work by Cannon and Britton (1926) many investigations have shown that the adrenals release catecholamines during exercise (*cf* Ashkar *et al* 1968) In this respect it is interesting that prolonged physical training in our study also significantly increases the total amount of adrenaline in the adrenals This increase is highly significant if it is expressed as μg adrenaline/kg b w However due to the concomitant increase in adrenal weight the adrenaline concentration is unchanged This weight increase of the adrenals after prolonged exercise is probably mainly due to an increase in adrenal cortical tissue as a result of the general adaptation to the stress of training (Selye 1953) Thus the increase in total adrenaline of the trained rats might be due to an increased influence from the hyperactive adrenal cortex since it is known that adrenal medullary synthesis of adrenaline is dependent on adrenal corticoids (Wurtman and Axelrod 1965 1966 Wurtman 1966) Another factor influencing the adrenaline content might be an increased synthesis due to the increased impulse flow in the splanchnic nerves during the training periods

This increase in total adrenaline content of the adrenals of the trained rats might be a manifestation of an adaptation to increased demands upon the cardiovascular and muscular systems or an unspecific reaction to stress

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Acta physiol scand 1971 82. 209—217

From the Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden

Dopa-decarboxylase in Autonomic and Sensory Ganglia of the Cat

By

Ezio Giacobini[1] and Barbro Nore

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Abstract

Giacobini, E and B Nore *Dopa decarboxylase in autonomic and sensory ganglia of the cat* Acta physiol scand 1971 *82* 209—217

The cellular localization of enzymes involved in the synthesis and inactivation of biogenic amines is still a matter for discussion Dopa decarboxylase (DDC) is present in parts of the brain rich in noradrenaline (NA) (Kuntzman *et al* 1961) and in sympathetic adrenergic nerves (Anden *et al* 1964) Increase in DDC activity in rat sciatic nerves after ligation was reported by Dahlstrom and Jonason (1968) No clear cut correlation has however been established between the presence of monoamines and DDC in adrenergic neurons

Although monoamineoxidase (MAO) is not exclusively associated with adrenergic neurons, a high level of MAO activity is a specific attribute of them (Consolo *et al* 1968, Giacobini *et al* 1970 Giacobini and Kerpel Fronius 1970)

Similarly, little is known about the mechanisms which regulate an co-ordinate syn thesis and inactivation of transmitters between cellbody and terminals particularly when *de novo* synthesis of enzymes is involved

Deprivation of functional synaptic connections induces measurable changes in the level of both transmitter (noradrenaline NA and inactivating enzyme (MAO) in the cell body of adrenergic neurons Giacobini *et al* 1970) Selective and reversible destruction of peripheral adrenergic terminals by means of 6 hydroxydopamine or

[1] Present address Research Lab AB Draco Fack S-22101 Lund

release of NA by reserpine produces an increase in the level of the synthesizing enzyme tyrosine hydroxylase in the cell body (Mueller *et al* 1969, Thoenen *et al* 1969, Thoenen *et al* 1970)

These observations indicate that the level of activity of enzymes related to intra neuronal mechanisms of synthesis and inactivation of transmitters in autonomic neurons may correlate well to the a) level of the transmitter in the neuron, b) func tional situation in that particular *neuron*

The level of activity of intraneuronal enzymes connected with chemical transmission is not in a steady state but represents probably one of the mechanisms participating *in the physiological regulation of the turnover of the transmitter in the neuron. It can* therefore be modified by one of the following mechanisms, a) change or suppression of the synaptic input on the neuron (Giacobini *et al* 1970), b) alteration or destruction of the nerve terminals (Mueller *et al* 1969, Thoenen *et al* 1969, Thoenen *et al* 1970) *This emphasizes the importance of estimating intraneuronal changes in levels* of enzymes connected with chemical transmission Preferably, an enzymatic study of this type should be able to give information about changes of enzymes in the different parts of the neuron, including subcellular structures that is to analyze selectively the activity in the cell body axon and synaptic terminals

Because of its complexity the central nervous system does not lend itself to this kind of detailed study We have therefore preferred to make our observations on the autonomic and sensory ganglia There is no reason to believe that the mechanisms of regulation studied in the ganglion cells should be different from those acting in the central nervous system

In this study the activity of the enzyme DDC *participating in the* intraneuronal synthesis of catecholamines was measured in homogenates of autonomic and sensory ganglia of the cat both in normal conditions and after preganglionic denervation of lumbar sympathetic ganglia In order to interpret postdenervation enzymatic changes at the pre and postsynaptic site of the ganglionic junction the level of enzymes participating in the synthesis and inactivation of catecholamines was related to the level of transmitters (acetylcholine ACh, and NA) in the same ganglion

The necessity of studying changes of DDC activity in single ganglia weighing a few milligrams stimulated our interest to develop a quantitative micromethod having an adequate sensitivity The method is described in this paper

Materials and methods

Preparation of the ganglionic material

Adult cats of both sexes were anesthetized with pentobarbitalsodium (40 mg/kg i p) About 45 min were necessary in order to remove the whole series of autonomic and sensory ganglia. The ganglia were placed in physiological saline at 0° C The connective tissue capsule was [illegible] and the ganglia were carefully cleaned under the dissection microscope The ganglia [illegible] with [illegible] as 20 [illegible] eigh-

The supernatant was usually stored at –70 C over night

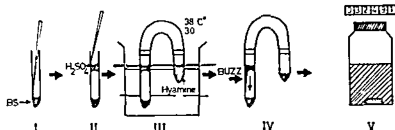


Fig 1 Schematic representation of the procedure for the radiochemical determination of DDC activity in μg samples of tissue For the description of the individual steps see text

The enzyme activity of the homogenate was unchanged after one month s storage at −70° C After 3 months the activity decreased by 20 %

Denervation procedure

Denervation of the lumbosacral ganglia (S_1 L_7 L_6) was performed under anesthesia and in aseptic conditions according to the technique of Buckley *et al* (1967) The part of the ganglionic chain resected included L_4 and L_5 ganglia as well Denervated ganglia were assayed for enzyme activity 4 or 7 weeks after the operation.

Enzymatic assay

a) Reagents

All the reagents were of analytical grade DL 3,4 dihydroxyphenyl alanine carboxyl ^{14}C (DOPA) was obtained from the New England Nuclear Corporation with a specific activity of 2 45—3 45 mC/mmole

Buffer substrate solution was freshly prepared for each experiment with the following composition DOPA ^{14}C 1 0 mM, pyridoxal phosphate 0 5 mM, L(+)-cysteine chloride 5 0 mM BSA (bovine serum albumine) 0 2 % phosphate buffer 83 mM, pM 7 4

b) Procedure

In a pointed microtube (length about 30 mm external diameter 4 0 mm internal diameter 2 5 mm) 1 1 μl of homogenate was mixed with 5 6 μl buffer substrate (Fig 1 I)

A ringlet of 18 8 μl of 2 5 M H_2SO_4 was placed 25 mm above the buffer substrate level (Fig 1 II) Th[illegible] an
other microtube [illegible] di
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The two tubes [illegible] 30
min at 38° C (Fig 1 III)

[illegible]

liquid scintillation spectrometer and corrected for 100 % efficiency The counting efficiency was of the order 93—95 % The calculation of the enzyme activity was done on the basis of the known specific activity of ^{14}C DOPA Experiments using $NaH^{14}CO_3$ showed that hyamine absorbed CO_2 to about 100 %

c) Control experiments

Triplicate control determinations were made with each set of analys s Four blanks containing boiled homogenates were included in each experiment

Ganglionic homogenates which were previously boiled did not show any activity

d) Inhibitors

The compound NSD 1015 (m hydroxybenzylhydrazine) inhibited DDC-activity by 100 % at a concentration of 10^{-6} M NSD 1034 (N m hydroxybenzyl N methylhydrazine) at 10^{-5} M

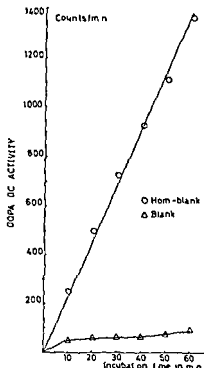


Fig 2

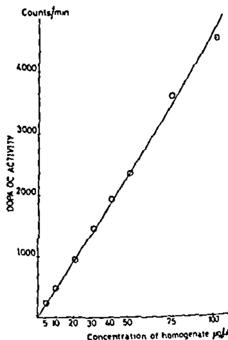


Fig 3

Fig 2 Time course of DDC activity by cat stellate homogenates (20 $\mu g/\mu l$)

Fig 3 Relationship between the concentration of ganglia homogenates in the range 5—100 $\mu g/\mu l$ and DDC activity

Comments on the method

Fig 2 shows the time course of CO_2 evolution by ^{14}C DOPA in cat stellate ganglia homogenates (20 $\mu g/\mu l$) and in blanks The curve demonstrates a linear relationship during the *first 60* min

Fig 3 shows the relationship between the concentration of ganglion homogenates in the range 5—100 $\mu g/\mu l$ and the DDC activity

DOPA easily undergoes spontaneous decarboxylation This was prevented by adding cysteine (Green *et al* 1945) This procedure was found to lower the blank from 400 CPM to 40 CPM without affecting the activity of the samples

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Sensitivity

The method allows 1×10^{-12} moles of CO_2/hr to be measured (calculated on the basis of a sample value twice the blank values)

TABLE I Dopadecarboxylase, monoamineoxidase activity and catecholamine content of autonomic and sensory ganglia of the cat

Type of ganglion	DDC activity[1]	MAO activity[2]	Catecholamines[3]	
			A	NA
Sup cerv[4]	14.4 ± 0.7 (18)	6.5 ± 0.3 (5)	0.12	10.2
Stellate	15.5 ± 1.1 (19)	6.6 ± 0.8 (6)	0.11	11.3
L_6	9.8 ± 0.9 (7)	4.2 ± 0.4 (11)		
L_7	11.0 ± 0.9 (17)	4.2 ± 0.3 (13)	0.85	7.2
S_1	11.4 ± 1.2 (4)	4.0 ± 0.3 (13)		
Celiac[5]	21.8 ± 1.1 (11)	11.1 ± 0.6 (6)	5.1	24.2
Sup mes.	17.9 ± 0.7 (7)	11.2 (3)	2.0	20.9
Inf mes	20.3 (2)	12.1 (2)	9.8	24.5
Ciliary	1.97 ± 0.24 (13)	2.05 ± 0.18 (12)	not measurable	
Nodose	0.16 ± 0.03 (13)	3.51 ± 0.28 (12)	not measurable	
Spinal	< 0.1[6]	1.47 ± 0.08 (16)	not measurable	

[1] [illegible]
[2] [illegible]
[3] [illegible]
[4] [illegible]
[5] [illegible] ficantly
[6] [illegible]

Results

The DDC activity of the sympathetic, parasympathetic and sensory ganglia investigated is presented in Table I and compared with MAO activity and catecholamines content in the same ganglia

As far as their DDC activity is concerned the sympathetic ganglia can be collected into three groups

Celiac and superior mesenteric show the highest activity, superior cervical and stellate are intermediate (celiac is significantly higher than stellate and superior cervical) and L_6, L_7 and S_1 ganglia show the lowest activity

Ciliary and nodose ganglia showed significantly lower DDC-activity than sympathetic ganglia Spinal ganglia (lumbar and thoracic segments) showed no measurable activity under the same experimental conditions

TABLE II Dopadecarboxylase, monoamineoxidase and catechol 0 methyltransferase activity in normal and preganglionically denervated L_7 ganglia of the cat

DDC activity[1]			MAO activity[2]		COMT activity[3]	
Normal L_7	Denervated 4 weeks	7 weeks	Normal L_7	Denervated 4 weeks	Normal L_7	Denervated 7 weeks
11 0±0 9 (17)	12 3±1 0 (4)	10 2±1 2 (4)	4 2±0 3 (13)	4 1±1 1 (5)	0 059±0 004 (14)	0 068±0 005 (5)

[1] DDC activity expressed in μmoles CO_2 formed/hour/g wet ±S E M
[2] MAO activity expressed in μmoles of product/hour/g wet±S E M The values reported are from Consolo *et al* 1968
[3] COMT activity expressed in μmoles of product/hour/g wet±S E M The values reported are from Giacobini and Kerpel Fronius, 1969

There is a good correlation (Table I) between DDC activities, MAO activities and the catecholamine contents (particularly NA) of autonomic ganglia

Table II shows the DDC activity of normal and preganglionically denervated L_7 ganglia (4 and 7 weeks after denervation) For comparison the MAO and COMT activities are also reported

Preganglionic denervation did not significantly change DDC MAO or COMT activity of L_7 ganglia

Discussion

Variations of DOPA decarboxylase activity in normal autonomic and sensory ganglia

Previous studies indicate that levels of activities of DDC may play a role in regulating brain concentrations of biogenic amines (Bogdanski *et al* 1957, Kuntzman *et al* 1961) The investigations carried out so far on DDC have been performed on gross areas of the brain (Bogdanski *et al* 1957 Kuntzman *et al* 1961) or in nerves (Anden *et al* 1964) which contain highly mixed cell populations, terminals and nerve fibres

The present method allows measurement of DDC activity at the μg level of tissue and therefore gives a better assessment of the contribution of each of various cell types A close relationship is apparent when the DDC activity values MAO activity and NA levels in different autonomic ganglia of the cat are compared (Table I) and three groups of ganglia can be distinguished

The ciliary ganglion belongs to the low activity group (DDC activity is only 1/10 of the celiac) while the celiac and mesenteric ganglia represent the high activity group

MAO activity and NA levels follow this trend very closely and show that in cat autonomic ganglia the synthesis inactivation and level of transmitter are closely related to each other

This is in agreement with the results of Kuntzman *et al* (1961) who found that with the exception of the caudate nucleus the capacity of different parts of the cat brain to form dopamine is proportional to the level of NA

Pscheidt and Haber (1965) in the chicken central nervous system and Håkanson and Owman (1966) in the pineal gland of several vertebrates did not find such a correlation Håkanson and Owman (1966) concluded that in the pineal gland DDC activity was mainly non neuronal (*i e* parenchymal), but they pointed out the fact that cat pineal is an exception as here the bulk of DDC (and MAO) activity appears to be present in the sympathetic nerves

The specific localization of DDC in adrenergic ganglion cells is also supported by the finding that DDC activity of parasympathetic and spinal ganglia is significantly lower or absent (Table I) than that of sympathetic ganglia which, like L_7, contain predominaly adrenergic cells (Consolo *et al* 1968) A low level of DDC activity is to be expected in the ciliary and the spinal ganglia as it is already known (Rexed and Euler 1951, Euler 1957) that sensory and parasympathetic nerves have a very low content of catecholamines and that only a moderate number of varicose nerve terminals coursing among the ganglion cells or surrounding the cell bodies are present in the ciliary ganglia of the cat, and fluoresce for catecholamines (Hamberger *et al* 1965 Owman and Santini 1966)

2 *Variations of DOPA decarboxylase activity after preganglionic denervation*

DOPA-decarboxylase activity measured 4 or 7 weeks after denervation is unchanged in L_7 ganglia (Table II) This indicates that DDC is probably not present in the cholinergic presynaptic structures of the sympathetic ganglia which are completely degenerated after denervation (Hàmory *et al* 1968) but is mainly if not solely con centrated postsynaptically in the adrenergic cell bodies and fibers The number of adrenergic cells in L_7 has been estimated to correspond to about 90 % of the total cell population (Consolo *et al* 1968)

Noradrenaline and ACh levels in sympathetic ganglia (L_7) vary in opposite direc tion after denervation NA increases by 30 % while ACh diminishes by 90 % (Fig 4) The explanation of this difference may be found in the different localization of the two transmitters in the ganglia Acetylcholine and cholineacetylase which are mainly present in the presynaptic endings are markedly hit by the degeneration and decrease considerably (Fig 4) Noradrenaline which is present in the cell body and fibres may on the contrary accumulate because of the slower rate of transport to the periphery following the lack of impinging synaptic stimuli (Giacobini *et al* 1970) Judging by our results on whole ganglion homogenates no parallel changes in the DDC activity are observed in the cell body

No significant changes after denervation are noticed in the whole ganglia experi ments as far as MAO or COMT activity is concerned (Fig 4) which might indicate the absence of these enzymes in the presynaptic site of the ganglia and their selective localization in cell bodies and fibres of adrenergic cells

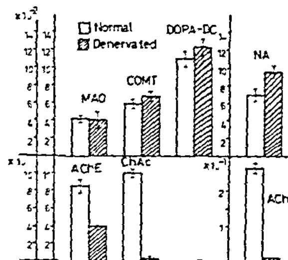


Fig 4 The effect of preganglionic denervation on five enzymes and two transmitters in L7 sympathetic ganglia of the cat (Giacobini 1971)

The presence of high levels of MAO and DDC-activity in the cell body and fibres of adrenergic neurons is very probable while the presence of COMT remains highly doubtful (Giacobini and Kerpel Fronius 1969

Dr R. Mc Caman Division of Neurosciences City of Hope Medical Center Duarte Cal., USA, Dr S Kerpel Fronius Department of Anatomy University of Budapest, Medical School Budapest, Hungary and Dr S Koslow National Institute of Mental Health Saint Elizabeths Hospital Washington D C USA are thanked for their advice and help in the methodological ..rt of this study

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Acta physiol. scand. 1971. 82. 218—228

From the Institute of Physiology and University Institute of Forensic Medicine
University of Oslo, Norway

Thrombocytes and Pulmonary Vascular Resistance during Hemorrhagic Hypotension

By

G Bo and J Hognestad

Received 19 November 1970

Abstract

Bo, G and J Hognestad *Thrombocytes and pulmonary vascular resistance during hemorrhagic hypotension* Acta physiol scand 1971 *82* 218—228

Prolonged hemorrhagic hypotension can induce an increase in pulmonary vascular resistance (PVR) In anesthetized cats with open chests and with positive pressure ventilation aortic blood flow pulmonary arterial left atrial and femoral arterial pressures were recorded Eight animals were bled until the mean arterial pressure was 50 mm Hg and this level was maintained for 3 hrs by further bleeding and/or small retransfusions The animals were then retransfused Another group of 5 animals were made thrombocytopenic by shunting their blood through a column of glassbeads These animals were then bled in the same way and kept hypotensive for the same period of time

During hypotension PVR in the first group increased gradually and by 370 per cent (mean value) In the thrombocytopenic group PVR increased only by 125 per cent (mean value) ($P<0.01$) The number of thrombocytes in arterial blood in the former group of animals decreased gradually by 37 per cent during the hypotensive period Only in 2 of the 8 animals in this group did examination with light microscopy reveal scattered thrombocyte aggregates in small pulmonary vessels

The thrombocytes thus seem to be involved in the development of increased PVR during hemorrhagic hypotension The fact that intravascular thrombocyte aggregates were not generally found may indicate that the thrombocytes act by other mechanisms than by simple vascular obstruction alone

Eaton Czebrinski and Smith (1945) discovered that the pulmonary arterial pressure in dogs rose to pre bleeding values or above during hemorrhagic hypotension This rise took place within an hour, and it occurred despite a reduction in cardiac output with a low rate of flow through the lungs, thus indicating an increase in pulmonary vascular resistance (PVR) In the lungs of the animals changes such as hemorrhage and edema were observed The same pulmonary vascular response pattern to hemorrhagic hypotension was observed by Cook and Webb (1968) In dogs subjected to 2 hrs of hemorrhagic hypotension they observed a 30 per cent rise in pulmonary arterial pressure (P_{PA}) and a 540 per cent elevation of PVR Measurements in humans have revealed similar marked elevations of P_{PA} after trauma and hemorrhage (Hardaway 1968) At the same time a reduction in the left atrial pres

sure (P_{LA}) has been observed, indicating that backward failure of the left ventricle does not prevail (Henry *et al* 1967) There is actually no agreement as to which factors are responsible for the pulmonary vascular responses to hemorrhagic hypotension Reflexes operating through the vagus nerve and/or the sympathetic nervous system might be involved Release and action of vasoactive substances such as serotonin, histamine, catecholamines or kinins may also occur

The aim of the present work was to obtain more information on the mechanisms interfering with the pulmonary vasculature during hemorrhagic hypotension In preliminary experiments on cats we noticed that a marked fall in the number of circulating thrombocytes occurred during the post hemorrhagic rise in P_{PA} Our main experiments therefore aimed at an analysis of the possible role of thrombocytes in the development of a high PVR subsequent to a severe blood loss A preliminary report of the present results has been published elsewhere (Bo and Hognestad 1970)

Methods

Animals Cats weighing 3—4 5 kg were anesthetized with i p injections (30 mg/kg) of sodium pentobarbitone (Nembutal®, Abbott)

The animal was placed on a heated operation table After the surgical procedures it was covered by a plast tent into which was led warm moist air Normal rectal temperature was thereby maintained

Surgical and experimental procedures Polyethylene catheters were introduced into both

lation was started

The chest was opened widely by a midsternum incision Precautions were taken to avoid hemorrhage by meticulous ligation of all bleeding vessels

A polyethylene catheter with a sidehole was introduced into the pulmonary artery through the wall of the right ventricle where it was anchored by a suture A polyvinyl catheter with sideholes was placed in the left atrium through an incision in the auricle to which it was sutured Both catheters were connected to pressure transducers for recording the pulmonary arterial pressure P_{PA} and the left atrial pressure P_{LA} respectively

Pressure recordings The various cardio-vascular pressures were recorded by Statham pressure transducers (P 23 Db and P 23 De) connected to a 6 channel recorder (Sanborn Model 320 Sanborn Co California)

cent of the full scale flow

The pulmonary vascular resistance PVR was calculated by the following equation

$$PVR = \frac{P_{PA} - P_{LA}\ (\text{mm Hg})}{\text{Flow (ml/sec)}}$$

For comparison of PVR values from animals of different weight and with different flow

set at 14 strokes per min The tidal volume varied from 40 to 60 ml and was adjusted so that the arterial P_{CO_2} was kept at about 30 mm Hg, which has been given as a normal value for cats (Sørensen 1967)

A ventilation overflow arrangement as described by Konzett and Rossler (1940) was used. The peak inspiratory pressure was usually 6—7 cm of water, but could be intermittently increased when one wanted to hyperinflate the lungs The expiratory pressure was set to 2 cm of water in order to prevent collapse of the lungs at the end of each expiration The overflow volume could be measured and the tidal volume computed The tidal volume/inspiratory peak pressure ratio was calculated as an indication of airway changes altering the pulmonary compliance

Hyperinflation of the lungs was carried out once every 30 min by increasing the peak inspiratory pressure up to 15 cm of water

Blood volume In most of the animals total blood volume was determined at the outset by a radioactive tracer dilution method (Hobbs 1967) The tracer used was ^{125}I tagged human

of hemorrhagic hypotension

Induction of thrombocytopenia Thrombocytopenia was induced by shunting circulating blood from a femoral artery to a femoral vein through a column of glassbeads The column consisted of 40—45 g of small glassbeads 0 5—1 mm in diameter in a plastic tube The beads were kept in place by a filter in each end of the tube Blood was pumped through the shunt at a flow of 25—30 ml/min with a Variable Speed Peristaltic Pump (Model 1210, Harvard Apparatus Co, Dover Mass U S A) After ten min the column was exchanged with a new and similar one which was used for another ten min

Thrombocyte counting in blood samples from the femoral artery was carried out according to the method of Brecher and Cronkite (1950) The pipettes were kept rotating in the pipette rotor for three min (Gruner 1970) Counting was done before and after the thoracotomy and then once per h during the experimental period

Blood gases and pH When femoral arterial blood samples were taken measurements of

Hematocrit values were estimated using an International microcapillary centrifuge MD

Histological examination The animals were killed by an overdose of Nembutal To prevent collapse of the lungs 4 % formol was poured into the bronchial tree The lungs were then immediately removed and fixed by immersion in Zenker's solution Specimens from all lobes of the lung were embedded in paraffin and sections 4 μ thick were cut The sections were stained with Hematoxylin Eosin and the MSB method of Lendrum *et al* (1962)

Results

Eight animals were subjected directly to hemorrhagic hypotension Three control animals underwent the same surgical procedure and received the same drugs as the others but were not exposed to the controlled hemorrhage Five other animals were made thrombocytopenic and then exposed to hemorrhagic hypotension Two further animals were exposed to hemorrhagic hypotension without preceding heparinization

The mean total blood volume of the animals was found to be 75 ml/kg body weight (±8 5 ml/kg) The average blood loss in the first group amounted to 31 per cent of the blood volume In the thrombocytopenic group the animals were on average bled 30 per cent of their total blood volume

In the 8 directly bled animals the P_{PA} changed in a characteristic way Fig 1

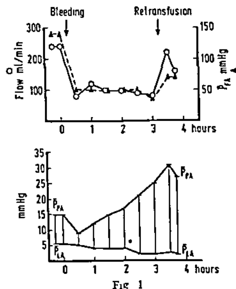


Fig 1

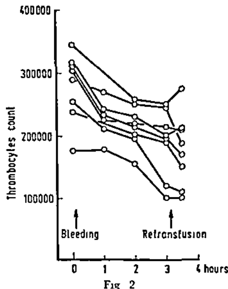


Fig 2

Fig 1 Development of mean pulmonary arterial pressure, P_{PA}, mean left atrial pressure P_{LA} cardiac output (mean flow) and mean femoral arterial pressure, P_{FA} during a 3 hr period of hemorrhagic hypotension in one single experiment

Fig 2 The number of circulating thrombocytes in arterial blood during a hypotensive period (mean arterial pressure 50 mm Hg) in eight bled animals which were initially non trombocytopenic

shows a typical experiment An immediate fall in the pressure was first observed as a result of the bleeding From about half an hour after start of the bleeding a continuous rise in P_{PA} took place and a value of 155 per cent of the initial pressure level was reached Throughout this rise in P_{PA} the aortic flow remained low and constant at about 40—50 per cent of its initial value When the animals were retransfused after 3 hrs of systemic hypotension, a sharp transient further rise in P_{PA} and an increase in aortic flow occurred The flow rose to its initial value in 4 animals in the others the rise was smaller

The P_{LA} declined gradually during the hemorrhagic hypotension from about 5 mm Hg down to 2—3 mm Hg There was a small rise in this parameter after re transfusion In none of the animals did P_{LA} rise above the initial value during the experimental period

Thoracotomy caused only negligible changes in the number of circulating thrombocytes During the hypotensive period a gradual and steady fall in circulating thrombocytes was found in all the 8 animals (Fig 2) The average fall was 37 ($\pm$25) per cent of the pre bleeding value

In the non bled control animals there were no appreciable changes in P_{PA} P_{LA} aortic flow and consequently in PVR during the observation period of 3 hrs During this period no fall in circulating thrombocytes was observed in this group of animals

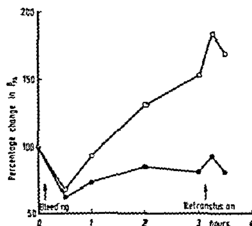


Fig 3

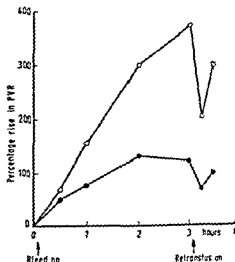


Fig 4

Fig 3 The percentage change in mean pulmonary arterial pressure P_{PA} during the 3 hrs of hemorrhagic hypotension The closed circles represent the mean values from 5 thrombocytopenic animals The open circles represent the mean values from 8 non thrombocytopenic animals

Fig 4 The percentage rise in pulmonary vascular resistance, PVR, during the 3 hrs of hemorrhagic hypotension The closed circles represent the mean values from 5 thrombocytopenic animals and the open circles the mean values from 8 non thrombocytopenic animals

In the 5 animals which had been made thrombocytopenic the development was different from that described in the non thrombocytopenic group of bled animals Shunting the circulating blood through the glassbead filters gave a mean fall in circulating thrombocytes to about 20 per cent of the initial value from 290 000 to 60 000 per mm³ on the average During the hypotensive period this group of animals showed a much more moderate change in P_{PA} and in PVR than did the other bled animals (Fig 3 and 4) The lower line of Fig 3 shows the development of the P_{PA} in the thrombocytopenic group The mean P_{PA} in this group did at no point climb above the pre bleeding value

Fig 4 gives the alterations in PVR in the thrombocytopenic and non thrombocytopenic groups When P_{PA} was at its lowest 1/2 hr after bleeding PVR was about 50—60 per cent above the pre bleeding value This rise was due to a smaller fall in pressure than in flow The maximal rise in PVR was 130 per cent above the initial value in the thrombocytopenic group as compared with a 370 per cent rise in the non thrombocytopenic group ($P < 0.01$) (Wilcoxon two sample test) The effect of retransfusion was a transient fall in PVR corresponding to a transient normalization of aortic flow

The hematocrit usually showed a fall after one h of hypotension in both bled groups After 3 hrs however, the hematocrit had risen up to the initial value or slightly above this In the non thrombocytopenic group the hematocrit thus rose from a mean pre-bleeding value of 37 per cent to a mean value of 39 per cent In the

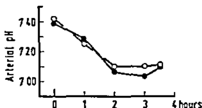


Fig 5 The mean arterial pH in two groups of animals during the 3 hrs of hemorrhagic hypotension Closed circles give mean values from the group of thrombocytopenic animals and open circles mean values from the group of non thrombocytopenic animals

thrombocytopenic group a mean value corresponding to the pre bleeding hematocrit value was reached

The ratio of tidal volume to inspiratory peak pressure fell by 24 per cent (mean value) in the non thrombocytopenic bled group, but showed an increase of 3 per cent (mean value) in the thrombocytopenic group

Metabolic acidosis was present in all animals of both groups The mean fall of arterial pH was 0 30 (Fig 5)

The arterial P_{O_2} which remained at about its pre bleeding level, was usually above 80 mm Hg, and the mean values were about equal in the various groups In a single experiment a value of 51 mm Hg was found at the end of the observation period

Heparinization of the animals might conceivably have altered the development of the pulmonary changes during hypotension In order to test this possibility 2 animals were exposed to the standard hemorrhagic hypotension without preceding heparinization The blood which was withdrawn was, however, heparinized (10 I U /ml), and small portions of this blood had to be injected at intervals in order to keep the blood pressure unchanged Protamine sulphate (0 1 mg/10 I U heparin) was, however, injected after each injection of heparin containing blood Both P_{PA} PVR, acidosis and number of circulating thrombocytes in these non heparinized animals developed in a way which was indistinguishable from what has been described above for heparinized animals exposed to hypotension

Light microscopy Sections from the lungs of animals from the main experimental group revealed an almost normal morphological picture Small foci of atelectasis and intra alveolar hemorrhage could be found in some of the sections however Scanty intra alveolar and perivascular edema was also found in some places In two of the animals platelet aggregates were found in small arteries and arterioles These aggregates extended into adjacent capillaries The lungs of the thrombocytopenic animals showed a normal morphological picture and no platelet aggregates were observed

Discussion

In the 8 non thrombocytopenic and heparinized animals which were bled the marked and progressive elevation of the pulmonary arterial pressure (P_{PA}) and the simultaneous fall in the left atrial pressure (P_{LA}) during hypotension represent a striking observation This gradually increasing perfusion pressure developed despite

a low flow through the lungs and reflects a considerable rise in the pulmonary vascular resistance, PVR

The pulmonary vascular system differs from the systemic one in several ways Not only is arterial pressure much lower in the former system but its vascular compliance is such that large increments in blood flow are evoked by small increments in arterial pressure (Fishman 1963) The use of a pressure flow ratio for recognition of vascular resistance changes thus requires that the ordinary passive pressure flow relationship is taken into consideration Evaluation of resistance changes is simplified if one uses either a technique of constant pressure with variable flow or a technique of constant flow with variable pressure When the situation in the pulmonary vasculature is evaluated during hemorrhagic hypotension however, both flow and pressure will alter The calculated PVR changes in our model are therefore in a complex way determined by both a passive hemodynamic factor and by factors involving alteration in vessel tone and/or in the number of perfused vessels

The effect of flow changes upon the pulmonary vascular pressure and resistance has been studied by several workers From experimental flow resistance curves (Fishman 1963) it appears that there will be a rise of about 50 per cent in calculated PVR when the flow through the lungs is suddenly halved One immediate result of bleeding in our animals was a reduction in the cardiac output by approximately 50 per cent The simultaneous fall in P_{PV} was however much smaller, and consequently the PVR rose considerably Half an hour after start of the bleeding PVR was about 60 per cent above its initial pre-bleeding value (Fig 4) This part of the PVR rise may be explained mainly as a result of the passive effect on the vasculature of the flow reduction The continued progressive rise in PVR during the hemorrhagic period must however largely be due to some other and active factors This rise in PVR was apparently the same whether or not the animals were heparinized

Two of our findings draw attention to the thrombocytes as playing a major role for the alterations developing in the pulmonary vascular bed during hypotension Firstly a constant fall in circulating thrombocytes was observed during the period of hemorrhagic hypotension. Secondly the thrombocytopenic animals seemed to be protected against the rise in PVR developing in the non thrombocytopenic animals

Pulmonary vessel occlusion by microemboli has been described after hemorrhagic hypotension in animals (Robb 1965) In such embolization thrombocytes have been discussed as possibly playing an important role The occurrence of a marked increase in the ability of the blood platelets to aggregate during and after surgery trauma or hemorrhage has thus been demonstrated (Dhall *et al* 1969) In dogs such an aggregability of the thrombocytes during hemorrhagic hypotension and after transfusion with stored blood has been suggested to result in widespread pulmonary microembolization with tissue damage and alveolar hemorrhage (Swank and Edwards 1968) Other investigators have demonstrated that labelled thrombocytes are preferably trapped in the lungs after trauma and hemorrhage (Ljungqvist Bergentz and Lewis 1970) In patients dead after major vascular surgery microemboli consisting of fibrin thrombocytes and blood cells are frequently found (Blaisdell *et al*

1966) Regional hypotension in dogs caused by clamping the aorta for three hrs has again been found to release a pulmonary syndrome similar to that described in humans exposed to a period of hypotension (Goodman *et al* 1968) Both light microscopic and electronmicroscopic examination then revealed microemboli containing blood platelets in pulmonary vessels

In all our non thrombocytopenic bled animals there was a tendency towards some thrombocyte aggregation after two and three hrs of hypotension Two three or more thrombocytes were often found aggregated during the counting procedure In the lungs from our animals vascular emboli were not a common finding however Scattered platelet aggregates were found in only 2 of the 8 animals developing a high PVR during hypotension Admittedly aggregated thrombocytes which have been trapped for a short period in small pulmonary vessels and which have then impeded pulmonary flow, may be difficult to demonstrate by light microscopy They may be easily washed out and lost during the fixation procedures Still the lack of such aggregates and the relative lack of pathological tissue changes such as edema and hemorrhage indicate that in the present experiments the thrombocytes must have acted also via mechanisms other than by total occlusion of vessels The observed pulmonary vascular responses to hemorrhagic hypotension may be similar in nature to that previously discussed for experimental pulmonary microembolism Such microembolisation is a strong stimulus to pulmonary vascular hypertension (Price Hata and Smith 1955) In this connection two mechanisms have been discussed in addition to simple mechanical obstruction of pulmonary vessels namely vasoconstriction induced by reflexes and direct vascular effects of released humoral substances (Price *et al* 1955 Halmagyi and Colebatch 1961 Levy Shapiro and Simmons 1969)

In our experiments and in those referred to above aggregated thrombocytes could conceivably have increased pulmonary vascular tone directly or reflexly via the release of vasoactive substances Blood platelets are known to contain a variety of such substances

Not much is known about possible reflexes acting upon the pulmonary vasculature during hemorrhage or similar conditions In our model one would presume the efferent limb of such reflexes to be sympathetic nerve fibres to the lung vessels Stimulation of the sympathetic nerves to isolated dog lungs at 50 imp/sec can induce marked increases in pulmonary arterial pressure (Allison Daly and Waaler 1961) When stimulation at 5—10 imp/sec is carried out vasoconstriction of pulmonary capacitance vessels is however apparently the predominant response Aarseth Nicolaysen and Waaler 1971) Only moderate increases in resistance are observed An increased activity in sympathetic nerve fibres to the lungs in our animals could conceivably be reflexly released from systemic baroreceptors Daly and Daly 1959 have thus shown that pulmonary vasoconstriction may be reflexly elicited in this way Stimulation of chemoreceptors pain fibres and other systemic structures could be important as well (Chien 1967) Moreover chemical or mechanical stimulation of receptive sites in or near the pulmonary vessels themselves may release reflex vasoconstriction (Whitteridge 1950 Waaler 1970

A striking finding in the present series of experiments was the lack of marked pathological alterations in the pulmonary tissue of the affected animals Henry and collaborators (1967) observed grave arterial hypoxemia along with atelectasis edema and hemorrhagic patches in the lungs in bled hypotensive dogs breathing spontaneously However, this hypoxemia and the pathologic changes in the lungs may be prevented or diminished by periodic hyperinflations (MacKay *et al* 1969) In accordance with these findings the type of ventilation used in our experiments *i e* periodic hyperinflations and some expiratory resistance, may have prevented not only the appearance of hypoxemia in the animals, but possibly also the development of pathological changes in the lungs

Hypoxia and particularly ventilation hypoxia is in itself a potent stimulus to pulmonary vasoconstriction The hypoxemia observed in our animals was moderate however, and could not have caused the observed marked rise in PVR (Hauge and Staub 1969) Furthermore the thrombocytopenic animals showed the same levels of Pa_{O_2} as the other group A lowering of arterial pH may also cause pulmonary vasoconstriction Severe acidosis did develop in our bled animals but again the pH values were similar for the thrombocytopenic animals which did not develop the marked increase in PVR

The simultaneous high rise in PVR and fall in compliance (tidal volume/inspiratory peak pressure ratio) in the non thrombocytopenic bled animals indicate that their airways as well as their vessels may have been reflexly or directly affected The unchanged compliance in the thrombocytopenic group of animals is noteworthy in this connection

It appears that some thrombocyte alterations are key events in the development of the pulmonary vascular changes during hypotension One would like to know then how such thrombocyte changes are triggered Catecholamines in certain concentrations have the ability to cause aggregation of human thrombocytes and release of their vasoactive substances *in vitro* (O Brien 1963 Bygdeman and Johnsen 1969) Also in the living rabbit adrenaline in physiological concentrations potentiates aggregation of thrombocytes induced by experimental pulmonary embolism (Thomas Gurewich and Kenneth 1968) During hypotension there occurs a marked release of catecholamines from the suprarenals Preliminary experiments in our laboratory seem to indicate that the release of catecholamines from the suprarenals during hemorrhagic hypotension is indeed connected with the thrombocyte-dependent rise in PVR described (Bø and Hognestad unpublished observations) Further studies along these lines are in progress

In conclusion therefore the gradual and marked increase in PVR seen during hemorrhagic hypotension in cats is apparently caused by some 'active' alteration in the pulmonary vasculature This alteration is again in some way dependent on the circulating thrombocytes which do not seem to cause total obstruction of small vessels but may well act via the release of some or all of the biologically active substances they are known to contain These substances may again directly or reflexly cause the increased pulmonary vascular resistance

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From the Department of Medical Chemistry University of Uppsala Sweden

Biological Evaluation of Protein Quality and Safety of a Lipoprotein Concentrate from Niger Seed* (Guizotia Abyssinica Cass.)

By

ANDERS EKLUND

Received 23 November 1970

Abstract

EKLUND, A *Biological evaluation of protein quality and safety of a lipoprotein concentrate from niger seed (Guizotia abyssinica Cass)* Acta physiol scand 1971 *82* 229—235

Growing rats were fed diets containing a lipoprotein concentrate prepared from niger seeds, as the sole source of protein Protein efficiency ratio (PER) and productive protein value (PPV) were calculated for two lev[illegible] respectively When the protein content of the [illegible]ding period of three weeks) The correspondin[illegible]eeding study was carried out on rats fed with [illegible] diet related changes were observed in the growth appearance, behaviour and survivals of the animals Hematological findings were normal The histopathological examinations of the main organs gave normal results Electron microscopy showed an increased amount of multi vesicular bodies (possibly lysosomes) as well as an increased content of fat droplets in the cytoplasm of liver cells The significance of these changes remains unclear

In a previous paper the preparation of a lipoprotein concentrate from niger seed was reported (Eklund 1970) Niger seed (*Guizotia abyssinica* Cass) more commonly known as nug (local name) is the main Ethiopian oilseed crop Many vegetable protein sources are known to contain toxic compounds *e g* hemagglutinins (Jaffe 1969), goitrogens (Van Etten 1969) and protease inhibitors (Liener and Kakade 1969) Therefore it is necessary to establish as far as possible that a preparation from any new protein source of vegetable origin is free from toxic components before it can be accepted as a constituent of human food (*cf* Protein Advisory Group 1970)

Recently some toxicological experiments with protein fractions from nug seeds were reported (Eklund and Ägren 1970 In the present study a biological protein evaluation of the lipoprotein concentrate from nug seeds was carried out and in addition the animals were examined by histopathological techniques after a 90 day feeding period

* CNU Report No 47 Children s Nutrition Unit Ethiopia)

Experimental

Materials The nug lipoprotein concentrate (NLPC) was prepared as previously described ... demy of Sciences—National Research ... New Jersey was used to prepare ... race were purchased from the Anti

Analytical methods The nitrogen content of diets was analysed by a macro-Kjeldahl method (Kungliga Lantbruksstyrelsens Kungorelse 1950) A nitrogen to protein conversion factor of 5 90 was used for the NLPC (Eklund 1970) For casein the conventional nitrogen to protein conversion factor of 6 25 was used The bo... *mined by the following procedure Each rat was k...* about 500 ml of conc H_2SO_4 for 5 hrs at 65° C ... to 1000 ml with conc H_2SO_4 Samples of 3 to 5 ... the macro Kjeldahl method

The fat content of diets was estimated by a Soxhlet extraction with diethyl ether (Kungliga Lantbruksstyrelsens Kungorelse 1950)

Biological evaluation The biological protein evaluation was carried out mainly according to Muller (1964) All rats were 24 days old at the beginning of the test Ten male rats of the same strain and age were sacrificed at the start of the feeding test in order to estimate the initial nitrogen content of the animals

The test included three diets One was a control diet based on casein and enriched with 0 5 % (w/w) of *DL* methionine (diet C) The protein content of this diet as eaten was 10 0 % The second diet contained NLPC as the sole source of protein at 11 1 % protein level (NLPC diet A) * The third diet contained NLCP as the sole source of protein at 22 4 % protein level (NLPC diet B) * The basal composition of the diets was according to Muller (1964) The fat content of NLPC (Eklund 1970) was sufficient to cover the oil requirement and NLPC containing diets were not supplied with more oil The NLPC diet B contained 10 % of fat while the two other diets contained 5 % For each diet ten male rats were used

The general behaviour of the animals was observed daily and registration of weight gain and food consumption was performed each week After three weeks five animals from each group were sacrificed and the nitrogen content of each was analysed (see above

The protein efficiency ratio (PER) was calculated according to the formula Weight gain in grams / Protein consumption in grams (*cf* Osborne *et al* 1919)

The productive protein value (PPV) (Muller 1964) was defined as (Body nitrogen (grams) at the end of the experimental period reduced by the Body nitrogen (grams) at the start/Nitrogen intake in grams) × 100 For discussions of the PER and PPV methods the reader may refer to National Academy of Sciences—National Research Council (*1963*)

The remaining animals five in each group were observed for a total period of 13 weeks after which time the animals were sacrificed At the same time blood from the abdominal aorta of the anaestesized animals was collected in plastic tubes containing 300 μl of 4 5 % (w/v) EDTA solution (trisodium salt monohydrate) at pH 7 4 as anticoagulant Determinations were made of hemoglobin hematocrit white blood cells differential count of white blood cells and thrombocytes Hemoglobin was analysed as cyanmethemoglobin (Bauer 1970) Hematocrit values were obtained by a micro method Centrifugation was accomplished in capillary tubes in a standardized high speed centrifuge (Adams Readacrit® micro hematocrit centrifuge Clay Adams Inc New York) Prior to leucocyte counting 25 μl of the blood sample was mixed with 475 μl of Turk reagent (0 02 % (w/v) gentian violet ... the blood sample ... citrate ... nt (Bostrom ... with May ... may be ob-

The weights of liver spleen kidneys adrenals gonads and heart were measured A histopathological examination was made on slices from the following organs liver pancreas small and large intestine kidneys adrenals gonads spleen heart lung and bone marrow

*The calculations of the protein contents of nug diets were based on an experimentally determined nitrogen to protein conversion factor (Eklund 1970) At the time when the two nug ... (5 32) Later on its was found that the correct ... tein contents of the two nug diets turned out ... 1 20 %

TABLE I Biological evaluation of NLPC with methionine enriched casein as a reference protein Ten male rats in each group

Diet	Protein source	Protein level (%)	Mean weight gain (g) per 3 weeks ±SD	Mean protein consumption (g) per 3 weeks ±SD	Mean PER per 3 weeks ±SD	Relative PER[1]	Mean PPV per 3 weeks ±SD[2]	Relative PPV[1,2]
A	NLPC	11 1	78 2±10 0	33 0±3 0	2 37±0 14	61 2	42 0±1 7	56.2
B	NLPC	22 4	111 5± 5 5	61 3±2 3	1 82±0 08	47 0	33 9±1 5	45 4
C	Casein + methionine	10 0	117 5±11 3	30 3±1 7	3 87±0 19	100 0	74 7±3 8	100 0
Degree of statistical significance[3]			A r C*** B r C not significant A r B***	A r C* B r C*** A r B***	A r C*** B r C*** A r B***		A r C** B r C** A r B**	

[1] The ratio for diet C was put at 100 0

microscope

Statistical methods Standard deviation (SD) was calculated according to Hoel (1966) The Student t test was used for the statistical evaluation of differences in weight gain (*cf* Hoel 1966) Statistical calculations of data on protein consumption PER PPV and relative organ weights were performed according to the Mann Whitney U test (Siegel 1956) The t test as well as the U test were used as two-tailed tests The degree of statistical significance was denoted according to Huitfeldt and Adolfsson (1969)

Results

In Table I the results from a three weeks feeding test are given The general behaviour of the animals was normal during this period None of the animals had any diarrhoeas or excess stools The NLPC diet A was consumed by the rats in almost the same quantities as the methionine enriched casein diet

The mean PER and PPV for the rats on the NLPC diet A (11 1 % of protein) were 2 37 and 42 0 respectively for the three weeks period The corresponding values for the rats on the methionine enriched casein diet were 3 87 and 74 7 The NLPC diet B (22 4 % of protein) gave lower PER and PPV values than the NLPC diet A

In order to detect toxic factors possibly present in the NLPC groups of five rats were fed on one of the three diets (A—C for a total period of 13 weeks

When fed on either the NLPC diet B or the methionine enriched casein diet the

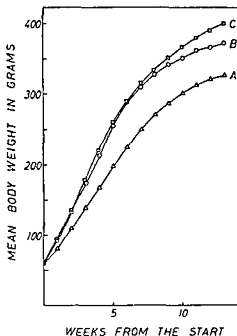


Fig 1

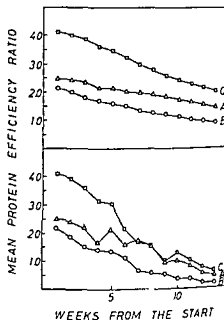


Fig 2

Fig 1 Growth of rats fed for 13 weeks on diets based on NLPC at the protein levels of 11 1 % (A) and 22 4 % (B) and methionine enriched casein at a 10 0 % protein level (C)

Fig 2 PER for rats fed diets based on NLPC at the protein levels of 11 1 % (A) and 22 4 % (B) and methionine enriched casein at a 10 0 % protein level (C) Mean PER was calculated for an accumulating number of weeks (upper figure) and for each one of the weeks (lower figure) During the first three weeks the values were based on 10 rats per group Thereafter each group contained 5 rats

rats had almost the same weight gain during the first 8 weeks (Fig 1) Then the weight gain of the rats in group B was slower than for the animals in group C The reason for this is not clear A possible explanation might be that a somewhat older batch of nug seeds was used for the preparation of the NLPC that was used in diet B during the last month of the experiment

Fig 2 illustrates how the PER changed during the feeding period As expected PER decreased when the rats became older and the growth rates decreased The fall in PER was more rapid in the group fed on methionine enriched casein The reason for this behaviour is not clear

During the fourth fifth and sixth week three of the rats fed on NLPC diet B got loose stools or even mild diarrhoeas of 5—10 days duration One rat in this group had two such periods of diarrhoea In the fifth week one of the rats fed on NLPC diet A had loose stools for about five days The rats fed on the methionine enriched casein diet never had excess stools During the last six experimental weeks all animals had normal stools With the exception of loose stools the animals did not show any sign of toxic reactions during the experimental time

TABLE II Hematological values obtained from rats fed on the test diets for 13 weeks

Test diet	Number of analysed rats	Hemoglobin g/100 ml Mean value, (range)	Hematocrit % Mean value, (range)	Number of white blood cells per mm³ Mean value (range)	Number of thrombocytes per mm³ Mean value, (range)
NLPC A	4	13.1 (12.8—13.6)	40.3 (40—41)	5900 (4100—7800)	753500 (720000—788000)
NLPC B	2	13.6 (13.1—14.1)	42.0 (41—43)	6850 (4000—9700)	713000 (652000—774000)
Casein + methionine	4	12.8 (12.0—13.5)	39.5 (38—42)	5900 (4200—8800)	679300 (581000—816000)

Blood analyses When the blood samples were collected some difficulties were observed in preventing the blood from forming microclots As seen in Table II the hemoglobin and hematocrit values as well as the number of white cells and thrombocytes were within the normal range for rats (Hardy 1967)

The rat blood samples contained few leucocytes and a large number of lymphocytes (Table III) which is a normal observation (Hardy 1967)

Postmortem findings No macroscopic changes were observed at the postmortem examination The corrected weights (in grams per 100 grams of body weight) of liver and adrenals were higher in the control group than in the groups receiving the NLPC diets (Table IV) Conversibly the animals fed on the reference protein showed lower weight figures for the gonads The weights of spleen and kidneys agreed relatively well between the groups B and C No significant differences were found in the relative weights of the heart

Histopathological examinations When slices from the different organs (see Experimental) were examined by light microscopy no pathological findings could be detected

Electron microscopy of the livers from animals fed on the two nug diets showed an increase in cytoplasmatic particles (multivesicular bodies) compared with the rats fed on the control diet These particles possibly were lysosomes An increased amount of fat droplets in the cytoplasma of liver cells from rats fed on the NLPC diet B was also observed Liver cells from rats fed on NLCP diet A contained much

TABLE III Differential count of white blood cells from the same rats as in Table II

Test diet	Number of rats analysed	Number of neutrophils per 100 white cells		Number of eosinophils per 100 white cells	Number of basophils per 100 white cells	Number of lymphocytes per 100 white cells	Number of monocytes per 100 white cells
		Rods	Segmented				
NLPC A	4	1—3	11—20	0—1	0	74—83	0—5
NLPC B	2	1	11—36	1	0	62—85	0—2
Casein + methionine	4	1—5	7—14	0—1	0	82—91	1—4

TABLE IV Relative weight of organs (in g per 100 g body weight) from rats fed on NLPC diet A NLPC diet B and the methionine enriched casein diet for 13 weeks The figures represent mean value and range from 5 rats

Group	Test diet	Liver	Spleen	Kidneys	Adrenals	Gonads	Heart
A	NLPC A	2 542 (2 358—2 674)	0 169 (0 151—0 196)	0 583 (0 548—0 613)	0 008 (0 007—0 011)	1 100 (1 038—1 156)	0 330 (0 298—0 355)
B	NLPC B	2 843 (2 613—2 943)	0 220 (0 212—0 231)	0 700 (0 660—0 757)	0 008 (0 006—0 009)	1 037 (1 004—1 082)	0 325 (0 310—0 340)
C	Casein+methionine	3 219 (2 974—3 621)	0 196 (0 170—0 214)	0 693 (0 650—0 726)	0 011 (0 009—0 012)	0 841 ((0 783—0 892)	0 312 (0 308—0 318)
Degree of statistical significance[1]		A v C** B v C** A v B*	A v C* B v C* A v B**	A v C** B v C not significant A v B**	A v C* B v C* A v B not significant	A v C** B v C** A v B*	A v C B v C A v B } not significant

[1] * 0 01 < p ⩽ 0 05 (probably significant)
** 0 001 < p ⩽ 0 01 (significant)

less fat droplets but yet a little more than found in the animals fed on methionine enriched casein In this connection it is important to point out that methionine is one of the limiting amino acids in NLPC (Eklund 1970)

Discussion

casein diet containing 10 % of protein and supplemented with 0 5 % of DL methionine was used as a reference in the biological evaluation of NLPC The figures of weight gain, PER and PPV for this reference diet seem to be equally high if not higher than for a diet based on whole egg (hen s) at the same level of protein (Ågren and Lieden 1970) If the PER and PPV for the methionine enriched casein diet were standardized to 100 0, the relative PER and PPV for the NLPC diet A were 61 and 56, respectively (see Table I) The classical chemical score (Block and Mitchell 1946) of the NLPC with reference to whole egg (Joint FAO/WHO Expert Group 1965) was estimated to 59 (Eklund 1970) Thus the figures for chemical score PER and PPV for the NLPC were rather close to each other

According to the foregoing it is clear that only negligible deviations from normal conditions were noticed during the 90 day feeding With one exception loose stools or diarrhoeas were observed only among the rats fed on the diet which contained the highest amount of NLPC (diet B) Since the general behaviour as well as the figures for food consumption and weight gain of the animals apparently were normal even during their diarrhoea periods this transient disturbance of bowel function does not seem to be particularly ominous

At the end of the period small discrepancies were found in the corrected weights for liver, adrenals, and gonads (Table IV) between the rats fed on the control diet

and those fed on the test diets There were also small changes in the morphology of the liver cells, observable only by electron microscopy These deviations could possibly be related to the low content of methionine (Eklund 1970)

It remains unclear if the changes discussed above have any pathological significance at all

This investigation was supported by grants from the *Tri Centennial Fund of the Bank of Sweden* to Professor G Agren and from the *Medical Faculty of the University of Uppsala*

I wish to express my gratitude to Professor G Agren for valuable discussions throughout the work The technical assistance of Miss B Kruse Mr B Ejdesjo and Mr J Soderman is gratefully acknowledged

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All hematological analyses were performed by Miss H Horberg

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From the Departments of Aviation and Naval Medicine, Faculty of Medicine, Karolinska Institutet, Stockholm, Sweden

Dynamics of Arterial Oxygen Tension in Response to Sinusoidal Work Load in Man

By

HILDING BJURSTEDT and OVE WIGERTZ

Received 27 November 1970

Abstract

BJURSTEDT, H and O WIGERTZ *Dynamics of arterial oxygen tension in response to sinusoidal work load in man* Acta physiol scand 1971 *82* 236—249

Dynamic characteristics of the response of arterial O_2 tension (P_{aO_2}) to supine submaximal leg exercise were studied in 7 physically active young men P_{aO_2} was recorded continuously in the radial artery at the wrist work load was varied sinusoidally between the extremes of 250 and 1050 kpm/min with periods of 0 75, 1 5, 3 0, and 7 0 min Time averaging harmonic analysis showed a clear dominance of the fundamental component over the second and third harmonics in the P_{aO_2} response, indicating approximately linear properties of the underlying system within the work load region studied The transfer function for P_{aO_2} exhibiting the characteristics of a variable regulated within narrow limits in the steady state condition indicated that the basic response of P_{aO_2} to a change in work load is a transient change in the opposite direction preceded by a pure time delay ... ance occurred for work load periods near 3 0 min (peak to-peak deviations approaching 14 ... Hg Referring P_{aO_2} changes to blood entering the left heart the estimated time delay before ... change in work load resulted in a change in P_{aO_2} amounted to 15 sec The existence of a resonance ... *interpreted in terms of an unbalance between factors determining O_2 uptake from* and supply to the alveolar space

Many investigations have included measurements of the arterial O_2 tension (P_{aO_2}) in steady state exercise under different conditions However, there are few reports concerning the dynamic behaviour of this variable in normal subjects Suskind *et al* (1950) have analyzed blood samples drawn from the radial artery over consecutive one-minute intervals They observed a fall in P_{aO_2} during the first and second minutes of constant load exercise followed by a slow return towards the pre exercise levels Matell (1963) and Barr *et al* (1964) who analyzed the time course of P_{aO_2} on the basis of consecutive half minute time-averages calculated from continuous recordings of arterial O_2 saturation and pH, reported an essentially unchanged P_{aO_2} during the first half minute of exercise, followed by a transient fall *In addition to* breath-to-breath fluctuations, the last-mentioned authors observed random changes with large amplitudes in the recorded variables These tended to obscure the basic response of P_{aO_2} to step changes in work load For better understanding of the role of P_{aO_2} in exercise hyperpnea further information is therefore required on the dynamic be-

haviour of this variable The primary objective of the present study was to estimate the dynamic parameters that characterize the behaviour of P_{aO_2} in man during un steady state exercise Time averaging harmonic analysis of continuous recordings of P_{aO_2} was employed to minimize the disturbing influence of random fluctuations in this variable and thus to reinforce its basic dynamic response

Methods

Seven healthy male students served as test subjects They were all well trained in endurance sports and engaged in similar training programs and phys cal activities Individual dimensional and functional data are given in Table I

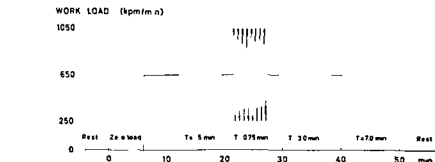
Ergometer

Precise sine wave work load patterns with preset amplitudes and frequencies were accompl shed by an electrically braked cycle ergometer (Holmgren and Mattsson 1954) modified and provided with a motor-driven cam and follower device as described elsewhere (Wigertz 1970)

Des gn of experiments

were chosen so as to be well tolerated by the subjects and to avo d any significant accumulation of lactic ac d in the arterial blood

Record ngs



protocol and during the zero-load cond t on

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From the Departments of Aviation and Naval Medicine, Faculty of Medicine, Karolinska Institutet, Stockholm, Sweden

Dynamics of Arterial Oxygen Tension in Response to Sinusoidal Work Load in Man

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Abstract

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Dynamic characteristics of the response of arterial O_2 tension (P_{aO_2}) to supine submaximal leg exercise were studied in 7 physically active young men P_{aO_2} was recorded continuously in the radial artery at the wrist, work load was varied sinusoidally between the extremes of 250 and 1050 kpm/min with periods of 0 75, 1 5, 3 0, and 7 0 min Time averaging harmonic analysis showed a clear dominance of the fundamental component over the second and third harmonics in the P_{aO_2} [illegible] the work load [illegible] interpreted in terms of an unbalance between factors determining O_2 uptake from, a[illegible] supp[illegible] to, the alveolar space

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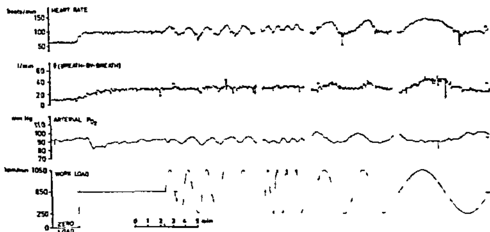


Fig 2 Segments of original recording from subject *HE* showing (from below) work load, arterial P_{O_2} recorded at left radial artery, ventilation on a breath-by-breath basis, and heart rate

the responses The number of cycles analyzed was 4, 2, 2, and 1 for T = 0 75, 1 5, 3 0 and 7 0 min,

analyzed by digital techniques using standard computer programs For a more detailed description and for formulas, see Wigertz (1970)

The primary P_{aO_2} signals were obtained from a catheter transducer-recording system, the response characteristics of which delayed and damped the intravascular P_{O_2} changes occurring at the catheter tip in the radial artery To reconstruct the latter changes the recorded data were corrected by digital computer technique using step-response curves of the recording system obtained prior to each experiment The computational algorithms used for mathematical recovery of the P_{aO_2} changes at the catheter tip are given in the Appendix

Results

Fig 2 shows segments from a chart record exemplifying the type of primary response signals that was used for data analysis The overall work performed was uniformly reported as light to moderate by the subjects The catheterization of the radial artery and blood sampling did not cause any signs of anxiety In general the resting heart rates were not high (Table II

Stable-state responses

The 6 min recordings of arterial O_2 tension P_{aO_2}, ventilation ($\dot{V}$) and heart rate (HR) at the constant work load of 650 kpm min served the primary purpose of

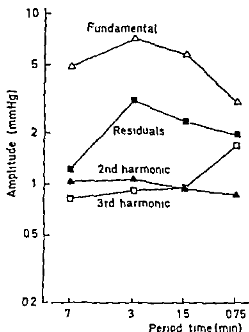


Fig 4 Amplitudes of fundamental, second and third harmonic sinusoidal components and re- [illegible] thus refer to the catheter tip in the radial artery Amplitudes of residuals are given in effective value × $\sqrt{2}$

Discussion

No previous attempt seems to have been made to employ sinusoidal work load to [illegible]dy the dynamic response of either end-tidal or arterial P_{O_2} The drive frequencies [illegible]sed in this study (the shortest period being 0 75 min) required the use of continuous recording technique to analyze the frequency response of P_{aO_2} This in turn required computer-oriented methods for correction of the dynamic distortion caused by the catheter-transducer-recording system Also it seemed worth-while to use such methods for estimation of central arterial P_{O_2} responses from those observed at the sampling site in the radial artery

The experiments were designed to avoid excessive exercise stress that might lead to variations in the dynamic characteristics of the variables under study For this purpose, the duration of the exercise portion of the protocol was limited to about 45 min Further, the subjects had a relatively high maximal working capacity (average predicted $V_{O_{2max}} > 4\ 6$ l/min), so that the overall mean of the work load including its sinusoidal portions, 650 kpm/min, and the peak value of the work-load sinusoids, 1050 kpm/min, corresponded to less than 33 and 53 % of the average maximal working capacity, respectively The necessity of withdrawing blood for the recording of P_{aO_2} produced a total blood loss of 250—260 ml over a total time of about 50 min Although this could affect the working capacity, it is not an amount usually expected to produce hemodynamic alterations in adults when the supine position is used to reduce gravitational effects This is in line with the observation of *Warren et al* (1945) who found no significant changes in cardiac output in normal supine adults following

removal of as much as 500 ml of blood In the present experiments, the magnitude of the mean upward drift in heart rate computed from the group-mean averages over the first and last drive frequencies of the experimental protocol ("d c components") was only 3 0 beats/min The largest individual increase in heart rate, 7 5 beats/min, was seen in subject HG who had the lowest predicted V_{O_2max} In this subject the peak value of the work load sinusoids corresponded to 72 % of the working capacity, and the possibility of a progressive, although slight increase in the blood lactic acid concentration can therefore not be excluded in this subject However, a slow upward drift in the heart rate of 7 5 beats/min over a period of 45 min is within the normal range observed by Ekelund (1966) in studies on individuals performing submaximal leg exercise in the supine position with no blood loss involved From this it may be inferred that the loss of blood entailed in the present study exerted little, if any, influence on the individual working capacity The mean d c component for P_{aO_2} showed a slow, progressive fall of 6 mm Hg over the exercise portion of the experimental protocol However, since P_{aO_2} was always measured at 37 0° C and since body temperature can be assumed to have risen by approximately 0 3° C during sinusoidal work load exercise, the true fall in the mean d c component for P_{O_2} was presumably a few mm Hg smaller (*cf* Bradley, Stupfel and Severinghaus, 1956) During the work load sinusoid with the largest period (T = 7 0) the peak-to-peak deviation in body temperature did not exceed 0 1° C

Estimation of dynamic parameters in the frequency response of P_{aO_2}

The P_{aO_2} response data, as presented in Fig 3, bear a rather close resemblance to those of a low order low pass filter with resonance characteristics and with an added pure time delay element A transfer function describing these features was therefore selected to be fitted to the observed data, with the objective of estimating the unknown dynamic parameters of this function The following transfer function was tested

$$H(s) = A^{*}_{ss}\exp(-sT_D)(1+s\tau_0)/\left(1+2\zeta\frac{s}{\omega_0}+\left(\frac{s}{\omega_0}\right)^2\right)$$

where A^{*}_{ss} is the asymptotic zero-frequency stable state amplitude T_D is a pure time delay, τ_0 determines the location of a zero ζ is the relative damping coefficient and ω_0 determines the undamped natural period $T_0 (= 2\pi/\omega_0$ and s is the Laplace notation of the complex frequency variable

The method used for mathematical fitting of the selected transfer function included measures to give adequate weights to response data from periods for which the times available for averaging harmonic analysis were not identical For parameter estimation the criterion selected involved minimization of the value of a loss function (Wigertz 1970) to obtain the best fit The fitting procedure was carried out by digital computer (IBM 360 75) The combination of parameter estimates that yielded the minimal value of the loss function (L is given in the upper row of Table III which also includes the standard deviations of the estimates The Bode diagram of Fig

5 *A* (upper and lower graph) presents the ensemble mean amplitude and phase lags in the frequency response of P_{aO_2} in the radial artery The curves in this diagram were constructed using the parameter estimates obtained as described and thus represent the best fit transfer function The characteristic features of this transfer function are a resonance peak in amplitude for periods near 3 0 min and a pure time delay of 29 sec (Table III) As will be developed further below, about half of this time delay can be accounted for by the lung to radial artery circulation time

The amplitude and phase lag points to the far left in Fig 5 *A* and *B* denote the estimated zero frequency response of P_{aO_2} to a change in work load corresponding to the amplitude of the work load sinusoids, *i.e.*, 400 kpm/min The amplitude (A_{ss}) of −3 0 mm Hg was obtained from the difference between the 6th min mean values for P_{aO_2} at 650 and 0 (zero load pedalling) kpm/min (*cf* Table II), *reduced to refer* to a 400 kpm/min change This estimate presupposes approximate linearity in the response of P_{aO_2} within the work load region 0—1050 kpm/min Such linearity is supported by the observed predominance of the fundamental component over the second and third harmonics in the region 250—1050 kpm/min Allowing for a slight increase in body temperature (0 2—0 3° C) the A_{ss} value would be overestimated by no more than 1 mm Hg It can be shown that a variation in the A_{ss} value of a few mm Hg has negligible influence on the location and magnitude of the resonance peak and on the T_D estimate

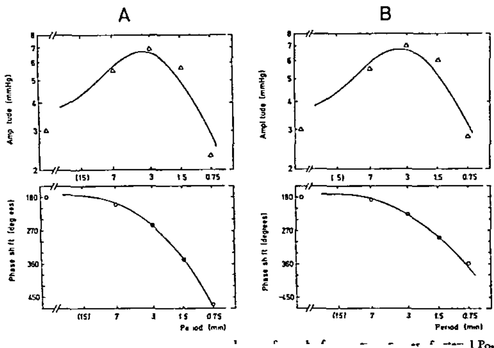
The best fit transfer function arrived at, was used to predict the time response of P_{aO_2} *to a step increase in work load from 0 to 650* kpm/min Fig 6 permits a com- between the experimentally obtained step response as referred to the samp site (A heavy line) and that predicted by analog simulation (B) As can be seen, ere is good agreement between observed and predicted time responses, notably with regard to pure time delay and time for maximal fall in P_{aO_2} This gives further *support to the assumption of an approximately linear* dynamic response of P_{aO_2} within the work load region 0 (zero load pedalling) — 1050 kpm/min

Central arterial P_{O_2} responses

The frequency response of P_{aO_2} has so far been referred to the relatively distant samp ling site in the radial artery at the wrist An estimated transfer function for circula

TABLE III Best fit parameter estimates for ensemble mean P_{aO_2} responses with ±1 *SD of the* estimates and minimal value of the loss function (L) The two sets of est mates refer to P_{O_2} responses at sampling site (catheter tip) in radial artery at the wrist and to the corresponding responses in blood leaving the lungs (central arterial P_{O_2}) n = 7

	A_{ss} (mm Hg)	A^*_{ss} (mm Hg)	τ_0 (sec)	T_D (sec)	ζ	T_0 (sec)	L
P_{O_2} in radial artery	−3 0	−3 4±0 6	124±55	28 8±1 8	1 0±0 2	199±27	0 0170
Central arterial P_{O_2}	−3 0	−3 4 _0 6	132±59	15 3±1 7	1 1±0 3	193±2[illegible]	0 0173



1 P_{O_2}

eart,

Amplitude and phase lag points at far left in diagrams signify estimated stable state responses (*cf* p 244) Curves in diagrams represent mathematical best fit transfer functions based on the parameter estimates in Table III

tory transport of blood from the pulmonary end-capillaries to the radial artery was used to obtain a rough estimate of the corresponding response in the blood leaving the lungs and entering the left heart (hereafter referred to as central arterial P_{O_2}) The required function was obtained from a separate series of experiments on 6 well trained subjects performing supine leg exercise at different work loads (Bjurstedt *et al* 1970) At 650 kpm/min which corresponded to the overall mean of the present work load sinusoids, the appearance and the mean circulation times from the lungs to the radial artery at the wrist averaged 12 and 15 sec, respectively Both parameters were found to vary inversely and approximately linearly with work load in the 250—1050 kpm/min region The 650 kpm/min transfer function was accordingly employed for the required correction, using the same method as that described in the Appendix for correction of instrument distortion The resulting frequency response data for central arterial P_{O_2} are plotted in the Bode diagram of Fig 5 *B* Using the same fitting procedure as previously described a new best fit combination of parameter estimates was obtained (Table III) It is of interest to note that the time-delay estimate (T_D) decreased from about 29 to 15 sec, the latter representing the delay after which a ch

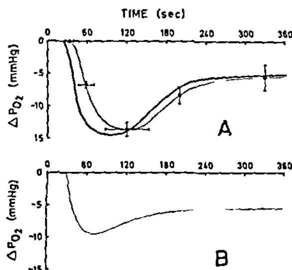


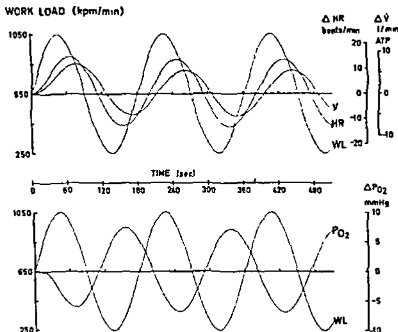
Fig 6 Comparison between observed (A) and predicted (B) time courses of P_{aO_2} following sustained step change in work load (0 → 600 kpm/min) at zero time
A *Thin line* average time course obtained by visual fitting to mean data from continuous tracings in 5 subjects (bars indicate ±1 SE); *Heavy line* corresponding curve after correction for dynamic response characteristics of catheter transducer recording system,
B Step response curve predicted from experimental frequency response data referred to sampling site in radial artery (generated by analog computer using

in work load was reflected by a change in central arterial P_{O_2} The curves in Fig 5 *B* were constructed on the basis of the new estimates and thus represent the best-fit transfer function for central arterial P_{O_2} A comparison with Fig 5 *A* shows that there was little change in the magnitude and location of the resonance peak in amplitude, the effect of the correction being more evident in the high-frequency region On the ·· is of the observation that the lung-to-ear circulation time amounts to only about 5 sec in moderate exercise (McIlroy *et al* 1966), it can be assumed that the dynamics of the P_{aO_2} changes near the carotid chemoreceptors were closely similar to those described by the Bode diagram in Fig 5 *B*

Interrelations of responses

It is well known that the adjustments of cardiac output to varying intensities of constant-load supine leg exercise are almost exclusive effected through changes in heart rate, except in heavy and exhaustive exercise This has recently been shown to hold also during the transient phases of supine leg exercise (Jones *et al* 1970), and for the purpose of this discussion the dynamic response characteristics of the cardiac output are therefore assumed to be approximately the same as described for the heart rate

The dynamic characteristics of both the ventilation and the heart-rate responses agree well with those in a recent study using sinusoidal load under closely similar experimental conditions and with a larger number of drive frequencies (Wigertz 1970) A statistical t test applied separately on observed *u*'s and *v*'s for identical drive frequencies showed no significant differences between the frequency-response data of the two studies, either for the ventilation or for the heart rate The finding that the response dynamics of these variables could accurately be described by first- and second-order exponential models, respectively, with negligible time delays and with the



$H_{\dot V}(s) = 12\ 7/(1+69\ 9\ s)$ l/min and
$H_{HR}(s) = 22\ 6\ \{0\ 63/(1+21\ 1\ s)+0\ 37/(1+147\ 3\ s)\}$ beats/min (Wigertz 1970),
$H_{P_{O_2}}(s) = -3\ 4\ \exp(-15\ 3\ s)(1+132\ s)\Big/\left(1+2.2\ \frac{193\,s}{2\pi}+\left(\frac{193\,s}{2\pi}\right)^2\right)$ mm Hg (present study), wher
s is the Laplace notation of the complex frequency variable

ventilation always lagging behind the heart rate (cardiac output), would therefore apply also to the present experiments

The present analysis has revealed what is apparently a characteristic response of P_{aO_2} to exercise The observation that the phase lag of this variable approached 180 degrees in the low-frequency region whereas its amplitude was largest at a higher drive frequency (resonance for periods near 3 0 min), indicates that the basic response of P_{aO_2} to a change in work load is a change in the opposite direction, and that with step changes in work load the response in P_{aO_2} appears as a temporary overshoot Assuming an essentially unchanged alveolar-arterial O_2 difference (*cf* Suskind *et al* 1950), the above observation also indicates that maximal unbalance between O_2 uptake from, and supply to, the alveolar space occurs for periods near 3 0 min, with the O_2 supply lagging behind the O_2 uptake This unbalance may in part be explained by the aforementioned lag between the cardiac output and the ventilation Fig 7 illustrates the time relations between ventilation, heart rate and central arterial P_{O_2} at T = 3 0 min Finally it is of interest that the transfer function for P_{aO_2} showed

band pass filtering properties often exhibited by regulated variables in physical closed loop control systems (the term "regulated' implying a variable held within narrow limits in the steady state condition)

The estimated 15 sec pure time delay for the central arterial P_O response suggests that the onset of changes in one or more variables critical for the dynamics of the pulmonary O_2 exchange occurs in the lungs some 15 sec after a change in work load Since the changes in both the ventilation and heart rate (cardiac output) exhibit negligible time delays (Wigertz 1970) and, furthermore, the alveolar arterial O_2 difference can be assumed to remain approximately constant, the most likely explanation for the estimated time delay seems to be that the a—$\bar{v}$ O_2 difference did not change appreciably for a corresponding period of time One explanation that presents itself immediately is that the 15 sec delay was due to the leg muscle to-lung circulation time An alternative interpretation is that part of this delay occurred as a result of a delayed onset of change in the local a—v O_2 difference in the working muscles

Appendix

Correction for distortion caused by the catheter transducer record ng system for P_{aO_2}

Let x(t) be the input signal y(t) the output signal and Δt the equidistant sampling interval (at which the signals are digitized) The following numerical difference equation may be set up

$$y(n\Delta t+T_D) = \sum_{i=0}^{r} b_i\, x(n\Delta t-i\Delta t) - \sum_{i=1}^{m} a_i\, y(n\Delta t-i\Delta t+T_D) \qquad (1)$$

n − 0 1 2

here T_D is pure time delay and a_i b_i are the coefficients (parameters) that characterize the system med to be of order m The equation expresses the value at each time $n\Delta t + T_D$ of the output s a weighted sum of m earlier output values and r input values T_D a_i and b_i may be estimated om corresponding input — output signals

The coefficients of the d fference equation (1) were estimated by means of a 'least squares meth od i e those values of the coefficients were searched which minimized a quadratic loss function' (L)

$$L = \sum_{n=1}^{k} [y_o(n\Delta t+T_D) - \sum_{i=0}^{r} b_i\, x(n\Delta t-i\Delta t) + \sum_{i=1}^{m} a_i\, y_o(n\Delta t-i\Delta t+T_D)]^2 \qquad (2)$$

It is assumed that the input is known from (1 r)Δt to $k\Delta t$ the observed output (y_o) from (1 m) $\Delta t+T_D$ to $k\Delta t-T_D$ The number of coefficients (m+r+1) of the system is unknown but may be estimated For computational methods see Åstrom (1968)

Provided that $b_0 \neq 0$ eq (1) may be rearranged to describe any input value as a function of the output and earlier input values

$$x(n\Delta t) \quad (y(n\Delta t+T_D) + \sum_{i=1}^{m} a_i\, y(n\Delta t-i\Delta t+T_D) - \sum_{i=1}^{r} b_i\, x(n\Delta t-i\Delta t))/b_0 \qquad (3)$$

... transd cer record ... s the ... ngs ... ter ... 1) is

used, putting

$x(t) = u_1 \sin\omega t + v_1 \cos\omega t$
$y(t) = u \sin\omega t + v \cos\omega t$

where u and v are the experimentally obtained harmonic variables and u_1 and v_1 the corrected variables to be estimated The following expression is obtained (a_0 1)

$$\sum_{i=0}^{m} a_i \left[u \sin(\omega(n\Delta t - i\Delta t + T_D)) + v \cos(\omega(n\Delta t - i\Delta t + T_D))\right] =$$

$$\sum_{i=0}^{r} b_i \left[u_1 \sin(\omega(n\Delta t - i\Delta t)) + v_1 \cos(\omega(n\Delta t - i\Delta t))\right] \tag{4}$$

$$u_1 = \frac{u(S_a S_b + C_a C_b) + v(S_a C_b - C_a S_b)}{S_b^2 + C_b^2} \tag{5}$$

$$v_1 = \frac{-u(S_a C_b - C_a S_b) + v(S_a S_b + C_a C_b)}{S_b^2 + C_b^2} \tag{6}$$

where $S_a = \sum_{i=0}^{m} a_i \sin(\omega(i\Delta t - T_D))$, $S_b = \sum_{i=0}^{r} b_i \sin(\omega i\Delta t)$

$C_a = \sum_{i=0}^{m} a_i \cos(\omega(i\Delta t - T_D))$, $C_b = \sum_{i=0}^{r} b_i \cos(\omega i\Delta t)$

Second and third harmonics were treated in a similar way

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From the Departments of Aviation and Naval Medicine, Faculty of Medicine, Karolinska Institutet, Stockholm, Sweden

Dynamics of Arterial Blood Pressure Responses to Sinusoidal Work Load in Man

By

Gunnar Rosenhamer and Ove Wigertz

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Abstract

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Systolic, diastolic, mean and pulse pressures in the radial artery, and heart rate in 7 physically active young men were subjected to frequency analysis by using sinusoidal work load as a forcing function Subjects exercised in the supine position on a cycle ergometer With the pedalling rate constant, the work load was varied sinusoidally over a range of drive frequencies between the two fixed extremes of 250 and 1050 kpm/min Predicted step responses obtained by applying the trans [illegible]

[illegible]rred for periods near 7 min, indicating overshoots for both variables in the time domain This behaviour suggests baroreflex inhibition as an explanation of an overdamped response of the heart rate The changes in radial diastolic pressure were small and not clearly related to the drive frequency The response of the radial mean pressure could not be described accurately by either first or second order functions, but exhibited phase lead features, suggesting sensitivity to rate of change in work load

The time course of changes in the arterial blood pressure in man following the sudden transition from rest to constant load exercise have been studied by several investigators (Holmgren 1956, Barr *et al* 1964, Linnarsson and Rosenhamer 1968) However, no quantitative information seems to be available concerning the dynamic properties that characterize the arterial pressure responses to exercise For the heart rate, the transient responses to the stimuli of both step changes and sinusoidal and ramp function changes in the work load have been recently reported from this laboratory (Wigertz 1970, Broman and Wigertz 1971, Karlsson and Wigertz 1971) It was found that the heart rate responded predominantly linearly to work load and that the dynamic heart rate/work load relationship could be modelled as a second-order transfer function

The present investigation, by using sinusoidal work load forcing, aimed at obtaining mathematical models defining the dynamic relationships between arterial pres-

sure components and work load At a constant pedalling rate on a cycle ergometer, the work load was varied sinusoidally between two fixed, submaximal levels and at different frequencies To minimize gravitational effects on the circulation, the experiments were carried out with the subjects in the supine position

Methods

Seven healthy male students were studied They were all competition cyclists engaged in similar

means of an analog computer (PACE TR 48) The mean pressure was obtained by low pass filtering The systolic and diastolic pressures were derived by peak follower circuits triggered from the pulse wave The pulse pressure was formed by electrical subtraction of diastolic from systolic pressure *Reference sine* $\left(\sin \frac{2\pi}{T} t = \text{work load profile}\right)$ and *cosine* $\left(\cos \frac{2\pi}{T} t\right)$ signals were generated conti

m n 150 mg Heparin (Vitrum) was given intravenously to prevent blood clotting He was then

TABLE I Dimensional and functional data

Subject	Age (yrs)	Weight (kg)	Height (cm)	$\dot{V}_{O_2max}$* (l STPD/min)
BA	21	66	179	5 3
SB	26	66	172	4 9
TC	22	76	190	4 9
PJ	23	82	183	4 6
KJ	21	75	178	3 2
OK	23	80	177	4 2
TS	18	59	174	5 3

* Predicted according to Åstrand (1960

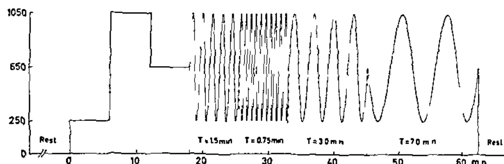


Fig 1 Time course of experimental protocol Zero-load is defined as loadless pedalling at 60 rpm Subsequent sinusoidal work loads (T being the period of a complete sine wave) were varied around a mean level of 650 kpm/min and with an amplitude (half peak to-peak) of 400 kpm/min

with a Heparin saline solution and recalibrated at least every 10 min Room temperature was 25± 2° C relative humidity averaged 50 % and barometric pressure 763 mm Hg

Computations The frequency responses of each pressure component were analysed by methods described in detail elsewhere (Wigertz 1970) Transient portions of the responses which followed the transition from one frequency to another were discarded using a trial and error method designed to satisfy the criterion that the error in the fundamental amplitude would always be less than

plitudes and phase lags were determined The contents of harmonics and noise in the pressure responses were analysed by digital computer techniques using standard computer programs (cf Wigertz 1970)

Results

The degree of exertion during the experiments was reported to be non strenuous by all subjects None of the subjects experienced distressing dyspnea or muscular pain during the 62 5 min supine exercise Fig 2 shows the type of recordings from which the primary data were obtained

Stable state (zero frequency) responses Individual and mean values for the heart rate and radial pressure components at rest and during the sixth min of exercise at 250 650 and 1050 kpm/min, respectively, are presented in Table II and III On the average, the radial mean pressure increased by 4 mm Hg following the transition from rest to 250 kpm/min, and changed little with 650 kpm/min At 1050 kpm/min it was 22 mm Hg higher than at rest The radial systolic and pulse pressures were both 15 mm Hg higher at 250 kpm/min than at rest, and 49 and 42 mm Hg higher, respec

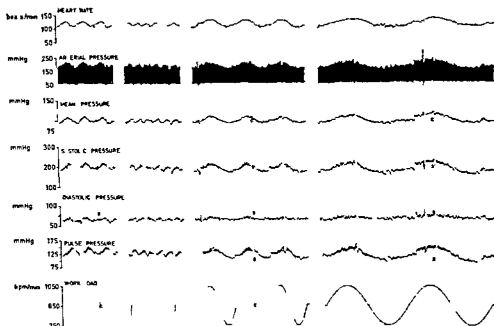


Fig 2 Segments of original recording from subject PJ Mean, systolic, diastolic and pulse pressures in the radial artery were derived from the arterial pressure signal by continuous analog computation

TABLE II Heart rate systolic arterial pressure and pulse pressure in radial artery at rest and during the sixth minute of constant load supine leg exercise of 250 650 and 1050 kpm/min

Subject	Heart rate (beats/min)				Systolic arterial pressure (mm Hg)				Pulse pressure (mm Hg)			
	Rest	250 kpm/min	650 kpm/min	1050 kpm/min	Rest	250 kpm/min	650 kpm/min	1050 kpm/min	Rest	250 kpm/min	650 kpm/min	1050 kpm min
BN	47	79	105	124	146	168	158	183	81	99	96	112
SB	59	88	106	129	142	156	168	199	69	85	97	119
TC	67	94	114	129	139	142	141	164	66	76	81	99
PJ	55	88	115	132	175	175	197	216	100	107	130	146
KJ	75	110	144	164	132	166	166	202	80	104	99	123
OK	49	83	121	140	134	146	157	199	69	83	96	125
TS	45	66	98	124	169	191	195	218	100	116	119	135
Mean	57	87	115	135	148	163	169	197	81	96	103	123

tively, at 1050 kpm/min The changes in the diastolic pressure were small and inconsistent at all work loads Stable state response amplitudes are defined as half the differences between the stable state responses to 1050 and 250 kpm/min and may be regarded as resulting from a sine wave work load of 0 frequency i e, with an infinite period From Table II and III the following group means for the stable state amplitudes (0-frequency responses to 400 kpm min) were obtained 17 0 mm Hg for the

TABLE III Arterial mean pressure and diastolic pressure in radial artery at rest and during the sixth minute of constant-load supine leg exercise of 250, 650 and 1050 kpm/min

Subject	Mean arterial pressure (mm Hg)				Diastolic arterial pressure (mm Hg)			
	Rest	250 kpm/min	650 kpm/min	1050 kpm/min	Rest	250 kpm/min	650 kpm/min	1050 kpm/min
BA	86	92	88	102	65	69	62	71
SB	92	94	97	113	73	72	71	80
TC	88	85	82	93	73	66	60	66
PJ	96	94	103	111	74	67	67	69
KJ	75	92	95	117	52	62	67	79
OK	82	83	88	110	66	64	61	75
TS	92	100	105	118	69	75	77	84
Mean	87	91	94	109	67	68	66	75

radial systolic pressure, 13 5 for the pulse pressure, 9 0 for the mean pressure, and 3 5 mm Hg for the diastolic pressure

Analysis of harmonics and noise The amplitudes of the fundamental, the 2nd and the 3rd harmonics, and the residuals (remaining harmonics and noise) were plotted for each period in the log-log-diagrams of Fig 3 *A* and *B* For all the variables the 2nd and 3rd harmonics and the residuals were rather constant in amplitude throughout the frequency range studied The amplitude of the fundamental decreased with increasing frequency, but dominated clearly over the 2nd and 3rd harmonics For the diastolic pressure the amplitudes of the residuals and the fundamental were of the same order of magnitude

Frequency responses and identification of dynamic parameters Because the fundamental of the frequency response showed prominence over the 2nd and 3rd harmonics for all pressure components (*cf* Fig 3), the further evaluation of their basic dynamic characteristics was focused on the behavior of the fundamental

Fig 4 *A* illustrates that the heart rate showed a continuous decrease in amplitude and an increase in phase lag with decreasing period The mean amplitude decreased from a stable-state value of 23 8 to 16 5 beats/min with the longest of the sinusoidal periods studied (7 0 min) This decrease was highly significant ($p < 0\ 001$) With the shortest period (0 75 min) the mean amplitude was reduced by as much as 79 % of the stable-state value The phase lag showed a maximal mean value of 66 degrees at this period Unlike for heart rate, group mean values for radial systolic (Fig 4 *B*) and pulse pressures (Fig 4 *C*) showed slight increments in amplitude above their stable-state values at the period of 7 min, *i e*, resonance characteristics at this period With further shortening of the period, the amplitude of both pressure components decreased below the steady-state value, as occurred also for the heart rate At the period of 0 75 min the mean amplitude for the systolic pressure was only 25 % of the steady-state value, and for the pulse pressure 22 %

For heart rate and radial systolic and pulse pressures, the polar plots in Fig 4 indicate dynamic characteristics that resemble those of a low-order, low-pass filter

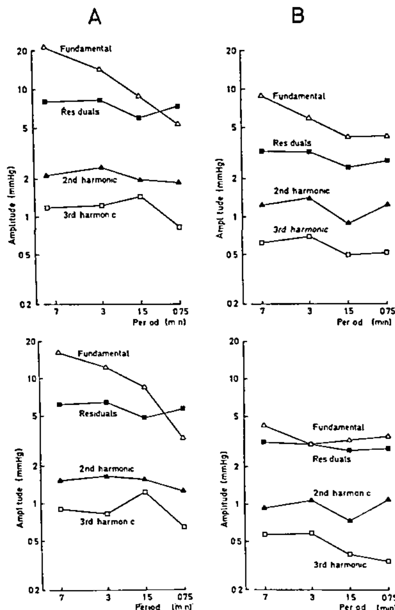


Fig 3 Amplitudes of fundamental, second, and third harmonic sinusoidal components and residuals (remaining harmonics and noise) versus period (log log scales) Mean values of 7 subjects

A radial systolic (upper panel) and pulse pressures (lower panel)

B radial mean (upper panel) and diastolic pressures (lower panel)

The fundamental and the harmonic components are given in peak amplitudes, residuals in effective value $\times \sqrt{2}$

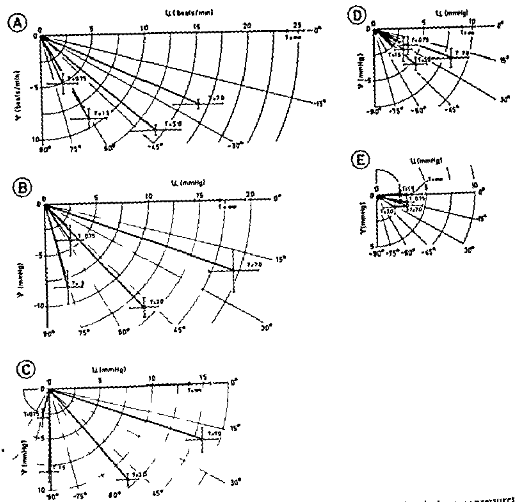


Fig 4 Polar diagrams of fundamental harmonic responses of heart rate and radial artery pressures Mean values for 7 subjects, bars denote ± SE

A heart rate
B systolic pressure
C pulse pressure
D mean pressure
E diastolic pressure

Families of vectors indicate amplitudes (vector magnitudes) and phase shifts (angle between vector and u-axis)

With the aim of estimating the unknown dynamic parameters of these variables it appeared justified, therefore, to select a low-order transfer function to be fitted to the experimental data The resonance characteristics of the systolic and pulse pressure responses indicate that their mathematical description might require a second-order rather than a first-order function For the mean pressure, on the other hand, the dynamics as shown in the polar diagram (Fig 4 *D*) appear more complex, and this is even more the case for the diastolic pressure (Fig 4 *E*) Thus the phase shift of the

mean pressure showed a decrease with the period at the two highest frequencies whereas the amplitude increased rather than decreased at the highest frequency. The changes in both amplitude and phase angle of the radial diastolic pressure were small, indicating a weaker frequency dependence than for the other pressure components. Also the standard errors of the variables in the polar diagram were considerably larger in relation to their absolute values than they were for the other pressure components. An attempt was made nevertheless to fit the mean and diastolic pressure responses to some low order functions as well.

The following first- and second-order transfer functions were tested for all variables with the objective of estimating their unknown dynamic parameters:

$$H(s) = A_{ss} \exp(-sT_D)/(1+s\tau) \quad (1)$$

$$H(s) = A'_{ss} \exp(-sT_D)(1+s\tau_e)/\left(1+2\zeta\frac{s}{\omega_e}+\left(\frac{s}{\omega_e}\right)^2\right) \quad (2)$$

[illegible] time delay [illegible] the

These functions were fitted to the experimental data by a procedure previously described by Wigertz (1970). This includes measures to give the individual data from each frequency (including the stable state values) adequate weights in the fitting process. Proper weighting is required because the variances of these data are inversely proportional to the total time at each frequency over which the time averaging analysis is made. Weighting was also performed to give prominence to the mean dynamic characteristics by suppressing the influence of inter-individual amplitude differences on the ensemble mean data. Parameter estimation involved the minimization of the value of a 'loss function' (hereafter referred to as 'L value') which yielded the best fit (Wigertz 1970).

The results of the first- and second-order fitting procedures for the weighted ensemble means of heart rate and the radial systolic and pulse pressures are given in Table IV and V respectively in the form of best fit values for the dynamic parameters. It is seen that by applying the second-order instead of the first-order transfer function ensemble mean L-values are lowered by as much as 13 9 times for the heart rate, 7 0 times for the systolic and 4 8 times for the pulse pressure. It is concluded

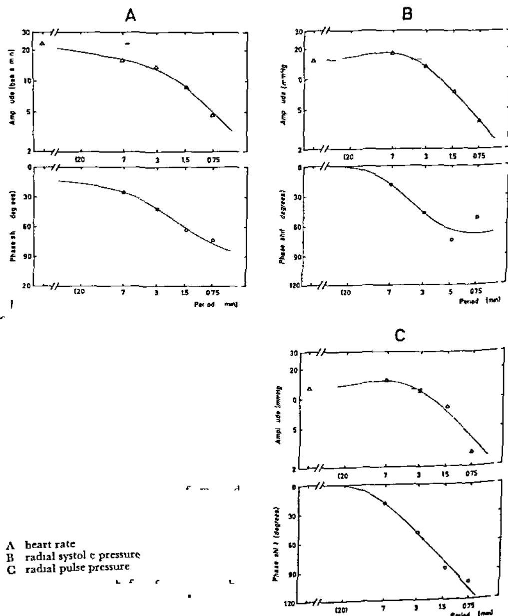
TABLE IV. Best fit parameter estimates ± 1 SD for weighted ensemble mean frequency response data and minimal values of loss function (L) first order transfer functions. Values refer to radial artery. n = 7

Variable	A_{ss} (beats/min) (mm Hg)	τ (sec)	T_D (sec)	$L \times 10^3$
Heart rate	19 3 ± 1 2	31 4 ± 4 8	−1 ? 2 4	4 588
Systolic pressure	18 1 ± 1 1	27 5 4 1	−0 1 2 ?	4 336
Pulse pressure	15 0 ± 0 9	2? 2 3 7	5 1 2 1	4 280
Mean pressure	7 0 ± 0 6	36 2 ± 7 5	−8 4 3 6	9 978
Diastolic pressure*	4 6 0 6	20 4 7 5	−4 6 ± 4 3	24 26?

* n = 4

TABLE V Best fit parameter estimates ±1 SD for weighted ensemble mean frequency response data and minimal values of loss function (L) second order transfer funct ons Values refer to radial artery n 7

Variable	A^{*}_{ss} (beats/min) (mm Hg)	T_D (sec)	τ_0 (sec)	ζ	T_0 (min)	$L \times 10^3$
Heart rate	23 2±0 7	0 5±0 8	160 3±61 5	1 76±0 22	7 4±1 6	0 331
Systolic pressure	15 3±0 9	−1 4±1 2	103 4±46 8	0 95±0 14	5 7±1 0	0 623
Pulse pressure	12 5±0 9	4 0±1 3	133 6±75 8	1 12±0 25	5 7±1 3	0 891



A heart rate
B radial systol c pressure
C radial pulse pressure

therefore that a function of the second order is required to give an accurate description of the heart rate and radial systolic and pulse pressure responses[1] The curves in Fig 5 *A*—*C* (Bode diagrams) signify the best fitting second order functions using the values of the dynamic parameters given in Table V To illustrate the degree of fitting the figure also shows the weighted ensemble mean amplitudes and phase lags



were approximately two and five times larger respectively than for systolic and pulse pressures (Table IV) For both pressure components second order fittings failed to converge

Discussion

For the heart rate the group mean time average (d c component for heart rate) over the last drive frequency of the experimental protocol was only 3 beats/min higher than the corresponding time average at the beginning of the experiment Further, the average radial systolic pulse, and mean pressures increased only by 4, 2 and 1 mm Hg respectively None of the periods tested had any consistent influence on the d c component of any of the variables studied These observations indicate that the pressure responses to a given sinusoidal load period were little influenced by prior sine load runs a change in the sequence of the work load sinusoids would therefore presumably not have significantly affected the average dynamic response characteristics of the variables under study

In one subject the peak load of 1050 kpm/min amounted to 76 % of his $V_{O_{2max}}$ There was no significant difference however in the harmonic analysis and mathematical parameter identification between values in this subject and those found in the 6 for whom the peak load amounted to less than 60 % of $V_{O_{2max}}$ This would suggest that the transfer functions obtained for the various pressure components are valid for a wide range of relative work loads

Even though the total duration of exercise was as long as one hour the fact that it was performed in the supine position should have minimized any gravitationally induced reduction in the stroke volume with time (*cf* Ekelund and Holmgren 1964) A time dependent decrease from other reasons such as an increase in body temper

[1] Applying statistical F test shows that at the 5 % level of significance and for the present degrees of freedom the hypothesis that the system under study is of the first order is accepted if

$$\frac{L_1 - L_2}{L_2} \cdot \frac{4}{2} < F_s(2, 4) = 6.94$$

or

$$L_1/L_2 < 4.47$$

where L_1 and L_2 are the L-values in the first and second-order cases respectively

ature, does not appear likely since the mean load (650 kpm/min) was relatively low in relation to the average working capacity (36 % of the $V_{O_{2max}}$, *cf* Ekelund 1967) Thus, the observed constancy of the group mean for the time average (d c component) of the heart rate should reflect a corresponding constancy of the cardiac output

It is well known that for mild to moderate intensities of constant-load exercise in the supine position, the stroke volume changes very little with the work rate It is believed accordingly that changes in stroke volume did not accompany the varying intensity of the leg muscle contractions at the sinusoidal work load profile Thus, the exercise induced heart-rate changes probably reflected proportionate changes in cardiac output

Estimation of central pressure responses Ever since the early investigations by Otto Frank (1905) it is well known that both the systolic and pulse pressures are augmented as the pulse wave is propagated towards the periphery (for reviews, see Hamilton and Dow 1939, Wetterer and Kenner 1968) The dynamic pressure/work-load transfer relationships obtained so far for systolic and pulse pressures are, strictly, valid only for the monitoring site in the radial artery at the wrist One therefore faces the question to what extent these relationships may be representative of those obtaining for corresponding pressure components in the proximal aorta (hereafter termed "central" pressures)

The transfer functions for central systolic and pulse pressures can be deduced provided the signal transfer characteristics of the aortic radial transmission link can be estimated There is evidence, that these characteristics can be approximated by a simple "gain" factor over the work load drive frequencies used Thus, Kroeker and Wood (1955) have demonstrated, by systematic, direct comparisons of simultaneously recorded central and radial arterial pressure pulses in 8 subjects, that the average radial pulse pressure was 1 46 times the central pulse pressure both at rest and during light to moderate leg exercise in the supine position The corresponding amplification of the systolic pressure in 12 subjects averaged 1 12 at rest and 1 13 during exercise From these findings, which appear to have been generally accepted (*cf* Wetterer and Kenner 1968), it can be assumed that the work-load range used in the present experiments did not affect the amplification of either of the two pressure components Further, there is no obvious reason for the amplification to change with the drive frequencies applied That this was not the case is supported by the fact that the contents of harmonics in the responses of the radial systolic and pulse pressures were low Warner (1957) demonstrated that pressure transmission in peripheral arteries is frequency-dependent, and presented the magnification ratio (amplitude distortion) for each frequency component of the pulse wave as it travels from the proximal aorta to the radial artery The finding of Kroeker and Wood (1955) that supine leg exercise does not alter the radial systolic and pulse pressures as percentages of the corresponding pressures centrally, suggests that the accompanying constriction of the resistance vessels of inactive arm muscles, which increases with the severity of exercise (Bevegård and Shepherd 1966), results in stationarity of the aortic to radial artery transfer characteristics when referred to the pulse-wave fundamental (heart rate) and each of its harmonics

TABLE VI Estimated zero-frequency amplitudes (A''_{ss}) in transfer functions for systolic and pulse pressures in radial and central arteries

	A''_{ss} (mm Hg)	
	Systolic pressure	Pulse pressure
Radial*	15 3	12 5
Central**	13 6	8 6

* From Table V
** Estimated values using gain constants obtained from Kroeker and Wood (1955) See further text on p 261

Hence, with the exception of the zero-frequency amplitudes (A''_{ss}), the dynamic parameters of the transfer functions for systolic and pulse pressures in the radial artery (eq (2) and Table V) should be representative also for the corresponding central pressure components The A''_{ss} value for central pulse pressure may be obtained by dividing the estimated A''_{ss} value for the radial artery by 1 46 (the value of the amplification factor given above), the central systolic pressure by instead using the factor 1 125 The resulting A''_{ss} values for the *central pressures are given in Table VI* As a corollary to the above reasoning, the normalized transfer functions for the radial systolic and pulse pressure responses should be representative also for the corresponding central pressure responses

The 250 → 1050 kpm/min normalized step responses of central systolic and pulse pressures, as predicted from the identified transfer functions describing the frequency responses of these variables, are shown in Fig 6, which also includes the predicted normalized step response of the heart rate An overshoot of both pressure components similar to the predicted ones in Fig 6 has been observed to occur in actual recordings of brachial artery pressures at the transition from one work load to a higher (Holmgren 1956), and also in radial artery pressure recordings following an abrupt change from rest to constant-load exercise of moderate intensity (Linnarsson and Rosenhamer 1968)

Since the second order fittings failed to converge for the radial mean and diastolic pressures, and the first-order fittings yielded relatively high L-values (Table IV) it is concluded that responses of these pressures to sinusoidal work could not be described with sufficient accuracy by linear, low-order transfer functions Consequently, no attempt was made to reconstruct the central mean and diastolic pressure responses

Interrelations of responses The measurements by Kroeker and Wood (1955) of the amplification of central systolic and pulse pressures in the radial artery were made with the opening of the aortic catheter directed against the blood flow Consequently, the velocity pressure ($^1{}_2\rho v^2$) was included in the pressure measurements Whereas this pressure component is negligible (less than 1 mm Hg in the radial artery with inactive arm muscles, it can become considerable in the aorta even during moderate leg exercise Further, if the fluctuations in heart rate are assumed to parallel those in

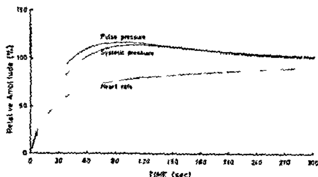


Fig 6 Computer-generated plots of 250 → 1050 kpm/min normalized step responses for heart rate, and radial systolic and pulse pressures obtained by applying "best-fit" normalized transfer functions estimated by time-averaging harmonic analysis of experimentally obtained responses to sinusoidal work load As outlined on p 261, the normalized plots are representative also for central systolic and pulse pressures Based on transfer functions given in eq (2) and Table V (pure time delays (T_D) omitted)

$$H_{HR}(s) = 23\ 2\,(1+160\,s) \,/\, \left(1+3\ 5\ \frac{7\ 4 \quad 60\,s}{2\pi} + \left(\frac{7\ 4 \quad 60\,s}{2\pi}\right)^2\right)$$

$$H_{SAP}(s) = 15\ 3\,(1+103\,s) \,/\, \left(1+1\ 9\ \frac{5\ 7 \quad 60\,s}{2\pi} + \left(\frac{5\ 7 \quad 60\,s}{2\pi}\right)^2\right)$$

$$H_{PP}(s) = 12\ 5\,(1+134\,s) \,/\, \left(1+2\ 2\ \frac{5\ 7 \quad 60\,s}{2\pi} + \left(\frac{5\ 7 \quad 60\,s}{2\pi}\right)^2\right)$$

where the subscripts HR SAP, and PP denote heart rate, systolic arterial pressure, and pulse pressure, respectively, and s is the Laplace notation of the complex frequency variable

cardiac output and hence those in the mean velocity of the blood in the proximal aorta, *the share contributed by the velocity pressure to the total pressure increases* in proportion to the heart rate squared This in turn means that, as the heart rate (cardiac output) *increases, there would be a progressively increasing difference* between the total pulse pressure centrally and the component thereof which distends the walls of the central vessels ("lateral" pressure) Consequently, the lateral pulse pressure would show a more marked overshoot than does the total pulse pressure In contrast to the systolic and pulse pressures, the time response of the heart rate is overdamped (Fig 6) *Reflex slowing of the heart is known to result from* stimulation of the arterial baroreceptors, this effect probably originating from increased pulse pressure rather than increased mean pressure (*cf* Heymans and Neil 1958) If so, the observation of an overshoot in the pulse pressure might, at least *in part*, explain the occurrence of an overdamped heart rate response If the velocity *component of the pulse pressure is not detected by the baroreceptors* (*cf* Marx *et al* 1967), a notion that can be questioned, then the overshoot of the lateral pulse pressure *would* have a stronger damping effect on the heart-rate increase than would be expected from the time response of the total pulse pressure

The radial mean pressure differed from both the heart rate and the systolic and pulse pressures in that its amplitude did not decrease *from the second* highest to the highest frequency, and its phase lag decreased rather than increased with *the two* highest *frequencies* (Fig 4) This "phase lead" suggests a sensitivity of the mean pressure to the rate of change in work load As the drive frequency *increases*, and the amplitude response of the heart rate decreases, the pressure pulse might widen *Close*

inspection of the curves showed this to be the case Such widening of the pressure pulse, which may occur as a result of diminished sympathetic discharge to the heart, would tend to raise the mean pressure, thus accounting for the rate sensitivity of this variable

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Acta physiol. scand. 1971. 82. 264—267
From the Department of Anatomy, University of Helsinki, Finland

Synaptic Connections of the Paracervical (Frankenhauser) Ganglion of the Rat Uterus Examined with the Electron Microscope after Division of the Sympathetic and Sacral Parasympathetic Nerves

By

T MUSTONEN and H TERÄVAINEN

Received 30 November 1970

Abstract

MUSTONEN T and H TERÄVAINEN *Synaptic connections of the paracervical (Frankenhauser) ganglion of the rat uterus examined with the electron microscope after division of the sympathetic and sacral parasympathetic nerves* Acta physiol scand 1971 82 264—267

The fine structure of the synapses in the paracervical (Frankenhauser) ganglion of the rat uterus was studied after sympathectomy and sacral parasympathectomy Degenerative nerve endings to the ganglion cells were observed after division of both the sympathetic and parasympathetic nerves No degeneration of the synapses to the chromaffin cells were seen The results are interpreted as evidence of both sympathetic and parasympathetic innervation of the ganglion cells which send their axons to the uterus whereas the chromaffin cells are innervated by intraganglionic nerves

The mammalian paracervical ganglion of the uterus is situated on either side of the uterus at the uterovaginal junction (Walter 1783, Frankenhauser 1876 and others cf Krantz 1959) Often, small paraganglia are also to be found The early accounts based on light microscopic studies (e g Frankenhauser 1876) described the connections of the ganglion with the sympathetic nerves originating from the hypogastric plexus with the sacral parasympathetic nerves and with the uterus and vagina In the rat the ganglion contains both adrenergic and cholinergic ganglion cells and clusters of small chromaffin cells

Electrophysiological studies have demonstrated the presence of a ganglionic synapse between the hypogastric and uterine nerves (e g Bower 1966) Although the early anatomical studies demonstrated connections between the parasympathetic nerves and the paracervical ganglion stimulation of the sacral parasympathetic nerves elicits no action potentials in the uterus (Bower 1966) and the existence of any synaptic connections with the paracervical ganglion remains to be shown

In degeneration studies at the light microscopic level it has been impossible to

discern synaptic connections between the sacral parasympathetic nerves (Teravainen and Mustonen 1969) or the hypogastric nerves and the neurones of the paracervical ganglion (Rosengren and Sjoberg 1967, Teravainen and Mustonen 1969) Owing to the obvious limitations of the light microscope it was decided to search for connections linking the paracervical ganglion with the sympathetic and sacral parasympathetic nerves at the ultrastructural level

Material and Methods

Sympathectomy was performed on 12 adult Spraque Dawley rats under ether anesthesia by careful thermocoagulation of all sympathetic nerve trunks and ganglia on both sides of the

Electron microscope observations were made from ganglia removed under light ether anesthesia 24 to 32 hours postoperatively and immediately fixed by immersion in 2 5 % glutaraldehyde buffered with phosphate at pH 7 4 at 4° C for 2.5 hrs (Sabatini *et al* 1963) The ganglia were postfixed in 1 % OsO_4 buffered at pH 7 4 with phosphate for 1.5 hrs and then dehydrated with a graded ethyl alcohol series The specimens embedded in Epon 812 (LUFT 1961) were sectioned and stained on grids with lead citrate (Reynolds 1963)

Results

Both the ganglion cells and the chromaffin cells in the normal paracervical ganglion were found to receive nerve terminals although the latter in substantially lesser amount The nerve terminals to the chromaffin cells contain both empty and dense-cored vesicles and exhibit intense catecholamine fluorescence (Teravainen and Mustonen 1969) being thus most probably aminergic The ganglion cells receive two types of nerve terminals one containing empty vesicles the other both empty and dense cored vesicles

Sympathectomy caused degeneration in some of the nerve fibres and nerve terminals within the paracervical ganglion all degenerating nerve endings being seen around the ganglion cells The fine structures of the degenerating synaptic terminals 24 to 36 hours after division of the nerve mainly showed a dense intensely osmiophilic mass of membrane containing material within the terminal axon (Fig 1) This amorphous mass made it very difficult to recognize the type of synaptic vesicles within it their discrimination often being impossible or uncertain At the 24 hour stage some of the degenerating axon terminals had a pale swollen matrix within which dense cored vesicles could easily be discerned (Fig 2) Sympathectomy did not cause degeneration in the synapses to the chromaffin cells

After sacral parasympathectomy even larger number of degenerative synapses were observed around the ganglion cells than after sympathectomy The difficulties in discriminating the type of vesicles in the degenerative nerves were much the same as following sympathectomy Parasympathectomy caused degeneration of the synaptic terminals which contained empty vesicles (Fig 3 In a few degenerating synaptic terminals some dense cored vesicles were observed among the usual empty synaptic



Fig 1 Degenerating axons (A) and axon terminals (AT) adjacent to the ganglion cell (G) of the paracervical ganglion 24 hours after coagulation of the sympathetic nerves Note the dark, intensely osmiophilic aggregations, consisting mainly of axonal vesicles and mitochondria in the degenerating nerve terminals, which renders it impossible to detect the type of synaptic vesicles within them Some non-degenerating axons (NA) are seen among the degenerating ones ×10 400

Fig 3 Only empty synaptic vesicles, and among them one swollen mitochondrion, are seen in this degenerating swollen axon terminal (AT) on the ganglion cell (G) 24 hrs after parasympathectomy Typically, the degenerating terminal axon is enveloped by glial cells ×12,000

Fig. 4. Two degenerating axon terminals (AT) on the ganglion cell (G) 24 hrs after parasympathectomy. Some mitochondria and empty axonal vesicles are seen among a few dense-cored vesicles (arrows). The membrane of the axon is fragmented and the degenerating axon terminal is enveloped by the glial cell in the typical way. ×13,600

vesicles after sacral parasympathectomy (Fig 4), and considering that parasympathetic nerves were selectively cut in the operation it thus seemed that sympathetic and parasympathetic nerves cannot be distinguished exclusively by the presence or absence of dense cored synaptic vesicles the relative amounts of these being the important feature Parasympathectomy caused no degeneration of the synapses to the chromaffin cells

Discussion

The present work provides an anatomical basis for the physiologically well known synaptic connection of the sympathetic hypogastric plexus with the neurones of the paracervical ganglion as demonstrated by electron microscopy An interesting observation was the presence of probable synaptic connections between the sacral parasympathetic nerves and the ganglion cells, although electrophysiologists have failed to observe response of the uterus to parasympathetic stimulation (*cf* Theobald 1968) Since stimulation of the pelvic nerves evokes no action potentials in the uterus (Bower 1966) the possibility remains that unlike the hypogastric nerves, the sacral nerves may be inhibitory in function

The fact that sacral parasympathectomy caused even more marked degeneration of the preterminal nerve fibres in the paracervical ganglion than did sympathectomy is probably due to the technical difficulty of destroying the sympathetic nerve plexus It seems likely that all the sympathetic nerves trunks were not sectioned in the operation There may, however, be numerous intraganglionic synaptic connections between the ganglion cells (Rosengren and Sjoberg 1967) Because no degeneration of the presumably aminergic synaptic terminals to the chromaffin cells was observed in the present work after either parasympathectomy or sympathectomy, it seems very likely that these cells are innervated by intraganglionic nerve fibres or by nerves consistently not sectioned

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Acta physiol. scand. 1971. 82. 268—271

From the Departments of Pharmacology, Psychology and Physiology, University of Goteborg Sweden

The Influence of the Nigro-Neostriatal Dopamine Pathway on Spinal Motoneuron Activity

By

NILS ERIK ANDEN, KNUT LARSSON and GORAN STEG

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Abstract

ANDEN N E, K LARSSON and G STEG *The influence of the nigro neostriatal dopamine pathway on spinal motoneuron activity* Acta physiol scand 1971 82 268—271

Reserpine treatment of rats with a unilateral lesion of the nigro-neostriatal dopamine neurons produced on the unoperated side a rigidity which could be demonstrated electromyographically in the calf muscles There was also a more marked increase in the α and a decrease in the γ-motoneuron excitability on the side contralateral than ipsilateral to the lesion indicating that the nigro-neostriatal dopamine neurons influence muscle tone by causing a shift of the balance between the α and γ motoneuron activity

Electrophysiological studies in rats have demonstrated an α rigidity in the parkinson like reserpine syndrome of akinesia rigidity and tremor (Steg 1964) The increased excitability of the α motoneurons appears simultaneously with a decreased γ-fibre activity These changes are due to a lack of one or several of the monoamines in the brain (Roos and Steg 1964)

A large uncrossed neuron system containing dopamine with the cell bodies in the substantia nigra and the nerve terminals in the neostriatum (the caudate nucleus-putamen) has been described (Anden *et al* 1964 Bertler *et al* 1964 Anden *et al* 1965 Anden *et al* 1966 a) Unilateral lesion of this nigro-neostriatal dopamine pathway followed by reserpine treatment produces a strong rigidity in the muscles on the side opposite to the lesion and a turning of the head and tail to the same side (Anden *et al* 1966 b These findings prompted us to study the relationship between the changes in α and γ motoneuron excitability seen after reserpine treatment and the inhibition of the neostriatal dopamine transmission produced by this drug

Material and Methods

Adult Sprague Dawley rats weighing 300—400 g were used The nigro-neostriatal dopamine pathway was unilaterally destroyed by electrolytic lesion (Hillarp 1947 in or immediately medial to the crus cerebri at the level of the corpus mamillare The operation was performed

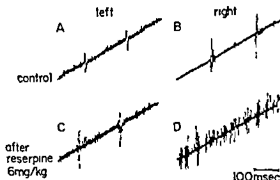


Fig 1 Changes in the electromyogram produced by reserpine in a rat with a lesion of the left nigro-neostriatal dopamine neuron system The electro-

80 *min after the administration of* reserpine (6 mg/kg i v) The increase in the stretch reflex response ("rigidity") after the reserpine injection was more pronounced on the right, unoperated side

about one month before the experiment The effect of the lesion on the nigro-neostriatal dopamine neurons was in all animals controlled after the experiment by determining the *in*

minals (*cf* Anden *et al* 1966 a)

The rats were laminectomized in halothane (Fluothane®) anasthesia. The L6 dorsal roots on

Results and Discussion

Before reserpine treatment, the animals demonstrated hardly any asymmetry at all Only a slight tendency to turn the tail to the operated side was constantly observed Also electrophysiologically, there were small or no differences in the electromyogram and in α- and γ-motoneuron activity between the two sides

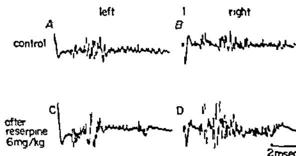


Fig 2 Changes in α and γ motoneuron activity produced by reserpine in a rat with a lesion of the left nigro-neostriatal dopamine neuron system The α and γ potentials in the left and right ventral L6 roots appeared after stimulation of the corresponding dorsal root The pictures were taken immediately before and 80 min after the administration of reserpine (6 mg/kg i v) The increase in the number of α potentials activated in the root reflex after the reserpine injection was more pronounced on the right unoperated side

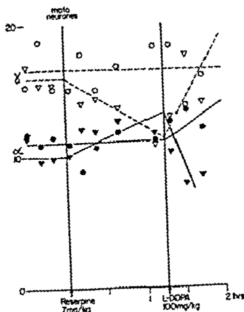


Fig 3 The effects of reserpine and L-3,4-dihydroxyphenylalanine (L-DOPA) on the root to root reflexes at the L6 level in a rat with a lesion of the left nigro-neostriatal neuron system Open symbols and broken lines indicate γ, filled symbols and continuous lines indicate α-reflex responses Left, operated side circles Right, unoperated side triangles Reciprocal changes in α and γ-motoneuron excitability on the side opposite to the lesion No distinct changes on the lesion side

After injection of reserpine (6—10 mg/kg i v), an assymmetrically distributed rigidity was demonstrated electromyographically as a greater increase in motor unit activity at rest and during dorsiflexion of the foot on the side contralateral to the lesion (Fig 1) This increase was linked to a higher amplitude of the α-motoneuron response to dorsal rooth stimulation (Fig 2) Usually this increase in α motoneuron activity was accompanied by a decrease in the γ motoneuron response Similar effects were observed after haloperidol administration (2 mg/kg i v)

The quantitative changes in α and γ-motoneuron responses to dorsal root stimulation are schematically illustrated in Fig 3 On the unoperated side reserpine produced an increase in the α- and a decrease in the γ-motoneuron discharge whereas very small or no changes were observed on the operated side An injection of the dopamine precursor L-3 4-dihydroxyphenylalanine (L-DOPA 100 mg/kg i v) restored more or less completely the motoneuron activity to the prereserpine level, indicating that the effect of reserpine was due to a functional catecholamine deficiency

The results of the present study taken togheter with those of a previous one (Andén *et al* 1966 b) show that the dopamine terminals of the neostriatum are involved in the control of muscle tone especially that related to posture Furthermore, it has been demonstrated here that this effect coincides with and therefore, in all likelihood, is due to the observed changes in the excitability of the α and γ-motoneurons

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Acta physiol. scand. 1971. 82. 272—276

From the Department of Zoophysiology, University of Uppsala, Sweden

Cyclic Variation of Body Temperature in Female Rabbits before and after Ovariectomy

By

J E Kihlstrom and C Lundberg

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Abstract

Kihlstrom, J E and C Lundberg *Cyclic variation of body temperature in female rabbits before and after ovariectomy* Acta physiol scand 1971 *82* 272—276

The body tempe [illegible] *months Three* of these rabbits [illegible] mperature the mean length of [illegible]

The observed [illegible] ppearing in two additional animals [illegible] significantly upon removal of the ovaries

The biphasic variation of the basal body temperature during the menstrual cycle, first observed by van de Velde (1904), has its correspondence during the oestrous cycle of many animals *e g* cows (Vollman and Vollman 1942), rabbits (Nieburgs *et al* 1946 Donnet *et al* 1960 a), long-tailed monkeys, rats, guinea pigs (Donnet *et al* 1960 b), and swines (Sanders *et al* 1964) This thermal variation is generally believed to be regulated by ovarian hormones as administration of progesterone is followed by a rise of the temperature (Palmer and Devillers 1939, Nieburgs *et al* 1946, Buxton and Atkinson 1948 Davis and Fugo 1948, Gianaroli and Moggian 1953, Wrenn *et al* 1958 1959) Moreover, the cyclic variation in the body temperature of women disappears during the climacteric (Vollman and Vollman 1942 Bergman 1950 Magallon and Masters 1950) and after ovariectomy (Palmer and Devillers 1939 Vollman and Vollman 1942 Davis and Fugo 1948, Bergman 1950)

In rabbits ovulation occurs only upon coitus or after artificial sexual stimulation Consequently, no corpora lutea is formed in absence of a male (Hill and White 1934, Myers and Poole 1962) and the secretion of progesterone is presumably very low It therefore, seems unlikely that the peak in body temperature during the sexual cycle of female rabbits would depend upon the thermogenic effect of this hormone Also in male rabbits there is a cyclic thermal variation (Degerman and Kihlstrom 1961) which is independent of gonadal hormones (Hornstein Kihlstrom and Degerman 1964) For these reasons we have studied the variation from day to day of the body temperature in female rabbits before and after ovariectomy

Materials and methods

have been used for the
delivered one or more
ely caged but could see
and water were always

ariectomized using
ial had to be killed
when the operated
were started again

The remaining nine intact does were used as control animals

Whether the temperature varies cyclically or not was studied by means of a serial χ^2 test, previously described by Jarnebrand and Kihlstrom (1969) Using this method the figures

Results

Statistically significant cyclic variations of body temperature were observed in 3 out of 18 intact animals (Table I) The length of these cycles varied from 6 to 11 days One individual (no 18) showed two cycles of different length, the longer one being a multiple of the shorter one Unfortunately one of these animals (no 10) had to be killed before being ovariectomized because of a febrile infection After gonadectomy cyclic variations were again observed in the same individuals and in two additional animals (Table I) During the same period no cycles were revealed in the control animals indicating that the cyclicity is not a seasonal phenomenon

The mean temperature of the intact animals was found to be 39.32 ± 0.22 (S D)° C that of ovariectomized animals 39.13 ± 0.19 The mean body temperatures of the control animals increased slightly during the experiments and were found to be significantly higher during the last than during the first third of the experimental period in seven out of nine animals (Table I On the other hand in six out of eight oophorectomized animals the body temperature was significantly decreased by the operation (Table I) No significant relationship between mean body temperature and cyclicity could be revealed

TABLE I Variations in body temperature of intact and *ovariectomized* rabbits

Group of animals	Rabbit No	Statistically significant cyclic variations					
		Cycle length, days	χ^2	p <	Cycle length, days	χ^2	p <
Control animals	1						
	2						
	3	No significant cyclic variation					
	4						
	5						
	6						
	7						
	8						
	9						
		Before ovariectomy			After ovariectomy (killed)		
Experimental animals	10	11	6 37	0 025			
	11	—	—	—	—	—	—
	12	6	3 84	0 05	7	7 26	0 01
	13	—	—	—	8	4 85	0 05
	14	—	—	—	—	—	—
	15	—	—	—	—	—	—
	16	—	—	—	—	—	—
	17	—	—	—	7	4 58	0 05
	18	7	10 09	0 005	3	6 84	0 01
		14	8 33	0 005			

Discussion

The present results confirm earlier observations (Nieburgs *et al* 1946, Donnet *et al* 1960) indicating that the body temperature may vary cyclically at least in some female rabbits The temperature fluctuations of female rabbits are comparatively small (see also Nieburgs *et al* 1946), and our methods may be too insensitive to register such variations in all animals As cyclicity occurred also after ovariectomy this cyclic variation can not be regulated by ovarian hormones However, the decrease of the mean body temperature upon oophorectomy indicates that the ovarian hormones may influence the mean level of the body temperature Thyroid activity has been shown to vary cyclically during the female sexual cycle with its maximum during oestrous in rats (Hunt 1944, Soliman and Reineke 1954 a, Feldman 1956), rabbits (Soliman and Ghanem 1956), ewes (Ghanem and Soliman 1956), buffaloes (Soliman and Said 1960), and cows (Soliman, Nasr and Zaki 1963) It is therefore possible that the cyclic variation in body temperature of the female rabbit may depend upon a corresponding variation in the activity of the thyroidea In women the cyclic variation in body temperature disappears after ovariectomy (Palmer and Devillers 1939, Vollman and Vollman 1942, Davis and Fugo 1948, Bergman 1950) A functioning ovary is also said to be necessary for the existence of the biphasic temperature curve in the cow (Wrenn *et al* 1959)

It may be important in this connection that the oestrous cycle of isolated female

Mean body temperature (±S D)		Statistical analysis		
First third of the experimental period	Last third of the experimental period	t	df	p <
39 45 ± 0 13	39 64 ± 0 42	1 833	36	0 05
39 41 ± 0 10	39 50 ± 0 15	0 912	52	0 20
39 32 ± 0 15	39 42 ± 0 15	2 294	46	0 075
39 25 ± 0 14	39 48 ± 0 13	5 814	46	0 0005
39 20 ± 0 11	39 41 ± 0 17	4 954	46	0 0005
39 47 ± 0 11	39 57 ± 0 21	2 002	46	0 05
39 20 ± 0 13	39 39 ± 0 15	4 583	46	0 0005
39 36 ± 0 14	*39 39 ± 0 23*	*0 533*	*46*	*0 30*
39 34 ± 0 08	39 43 ± 0 16	2 118	46	0 025
Before ovariectomy	After ovariectomy			
39 08 ± 0 17	*(killed)*	—	—	—
39 10 ± 0 26	39 04 ± 0 18	1 232	84	0 15
39 03 ± 0 14	38 93 ± 0 15	2 891	82	0 0125
39 39 ± 0 16	39 25 ± 0 17	3 550	73	0 0005
39 35 ± 0 18	*39 21 ± 0 15*	*3 691*	*72*	*0 0005*
39 32 ± 0 12	39 08 ± 0 19	6 383	72	0 0005
39 36 ± 0 11	39 14 ± 0 16	6 351	82	0 0005
39 29 ± 0 18	39 23 ± 0 14	1 492	72	0 10
39 25 ± 0 12	*39 13 ± 0 14*	*3 710*	*73*	*0 0005*

rabbits consists of a follicular phase only, no corpora lutea being formed in the absence of copulation and the mature follicles becoming atretic after having lasted for 4 to 10 days (Hill and White 1934, Myers and Poole 1962) Consequently, it may exist two different types of cyclic variation in body temperature, one regulated by gonadal hormones and occurring in animals having spontaneous ovulation, the *other type being independent of these hormones and occurring at least in some* rabbits

The slight increase in body temperature of the control animals during the experimental period may indicate a seasonal variation

If thyroid activity varies periodocally in both sexes such a variation may explain why the cyclicity in the body temperature of the male rabbit (Degerman and Kihlstrom 1964) persists after castration (Hornstein, Kihlstrom and Degerman 1964)

The mean temperature of intact female rabbits (39 32° C) is very close to that found in males (39 34° C Degerman and Kihlström 1964)

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From the Department of Physiology II Karolinska Institutet, and the Department of Ophthalmology, Karolinska Sjukhuset Stockholm, Sweden

Long-Term Changes in the ERG Following Transection of the Optic Nerve in the Rabbit

By

ERIK BORG and BENGT KNAVE

Received 16 December 1970

Abstract

BORG, E and B KNAVE *Long term changes in the ERG following transection of the optic nerve in the rabbit* Acta physiol scand 1971 *82* 277—281

The changes in ERG were studied in the rabbit for 22 months after transection of the optic nerve Immediately after the transection a small amplitude increase was found This increase was constant for the next 8 months Twenty two months after the section however, the amplitudes decreased The initial increase is interpreted as a functional sign of the existence of centrifugal activity in the optic nerve that decreases retinal excitability The final decrease in the ERG amplitudes is interpreted as a sign of retrograde degeneration in the retina

Changes in retinal function after optic nerve section might be due to interruption of efferent influences or to retrograde changes in the retina Although there is strong evidence for centrifugal fibres in the optic nerve in mammals (Granit 1955, Dodt 1956 Jacobson and Suzuki 1962, Spinelli *et al* 1965 Spinelli and Weingarten 1966, Weingarten and Spinelli 1966) their existence has been questioned by some authors (Brindley 1960 for further review, see Ogden 1968)

The retrograde degeneration in the retina following transection of the optic nerve has been shown to be a slow process which can not be verified histologically until about two years after the transection (monkey van Buren 1963 cat Stone 1965, puppy Horsten and Winkelman 1969)

Against the background of the slow retrograde degeneration and the possible efferent influence, the present work was performed in order to study the functional changes in the retina following optic denervation A recently described method was used which allows long term ERG studies on unanesthetized rabbits (Knave 1970 a b) In the present paper the changes in ERG during 22 months after optic nerve section will be described as well as the histological picture at the end of this time

Methods

Transection of the optic nerve Intracranial transection of the left optic nerve was performed in an adult pigmented Chinchilla rabbit under i.v. pentobarbital anesthesia A craniotomy was performed medial to the left orbit The dura was incised and by careful suction a hole was made through the limbic cortex just inside the lateral cranial wall Transection was performed

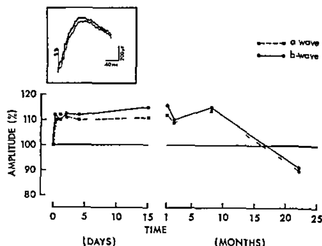


Fig 1 Amplitude changes in the ERG following transection of the left optic nerve of a rabbit. Amplitudes (a wave broken line b wave solid line) are expressed in per cent of those of the intact right eye Inset shows the normal rabbit ERG obtained in response to a bright electronic flash with an intensity of about 7 log units above the b wave threshold of the dark adapted eye DC amplification Records from right (upper tracing) and left eye (lower tracing) are similar in amplitude and shape

allowed repeated ERG recordings to be made during a long period of time in the non anesthetized rabbit As a result of strictly standardized conditions there were only small variations in the normal ERG amplitudes Recordings from both eyes illuminated with equal intensity, were made simultaneously The ERG amplitudes of the left eye were always compared to those of the intact right eye which served as a reference The normal variation in the relative differences between the right and the left eye has been investigated earlier the weighted s_{a_d} = 3 9 per cent and $s_{b_{diff}}$ = 5 0 pr cent (Knave 1970 a b) An electronic flash with an intensity of 7 log units above the b wave threshold was are required to elicit an adequate a wave in the rabbit responses from the right (upper tracing) and the left

Histological technique After anesthetizing the anim heparin added) the eye was fixed by transcardial perfus malin with the anterior segments 0 μ) in the horizontal plane and visualization of the cell bodies counting the cells in three cor e thickness of the nuclear layers was determined from microphotographs

Results

Amplitude changes in the ERG following transection of the optic nerve

The changes in the a and b wave following optic denervation are shown in Fig 1 The transection of the optic nerve was performed at zero on the horizontal time axis The ERG amplitudes (vertical axis) were expressed in per cent of the intact right eye The pre experimental relative amplitude value was adjusted to 100 per cent with subsequent correction of the experimental values A solid line between filled circles represents the b wave and a broken line between squares the a wave

In Fig 1 there was an immediate increase in the a wave as well as in the b wave, which varied between 10 and 15 per cent during the first 8 months after transection

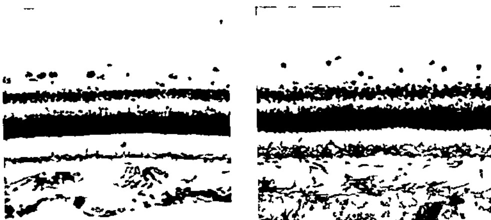


Fig 2 Cellular composition of corresponding portions of the central retina Normal eye (left) and the eye 22 months after optic nerve section (right) Cresyl violet stain

On the final recording 22 months after transection however a 9 per cent decrease was noted in both the *a* and *b* wave After this recording the rabbit was killed and the eyes were removed for histological examination

The immediate increase in the ERG amplitudes noted as soon as 4 hrs after the transection is statistically significant of both *a* and *b* wave ($p \leqslant 0.001$) The final decrease in the amplitudes 22 months after the transection was recorded twice with a week s interval The mean of the *a* wave was significantly below reference level ($p \leqslant 0.001$ and *b* wave was also significantly below reference level ($p \leqslant 0.01$)

Histological picture

The left optic nerve was found completely interrupted immediately peripheral to the chiasma Atrophy of the peripheral part of the nerve was not detected A representation of the cyto architecture of the retina in corresponding portions of the eye with cut optic nerve (right) and the normal eye (left) is shown in Fig 2 This figure illustrates that there is no obvious difference in the cellular composition of the two eyes The number of ganglion cells counted in three horisontal sections at equal distance from the papilla was only insignificantly less in the retina with the sectioned optic nerve The size of these ganglion cells appeared however generally smaller (see Fig 2) The thickness of the nuclear layers varied as a function of their position in the retina being less thick at the periphery Comparison of corresponding sections revealed no significant difference in thickness

Discussion

The initial increase in the ERG amplitudes that immediately follows transection of the optic nerve might be interpreted as functional evidence of the existence of centrifugal fibers that decrease retinal excitability (Jacobson and Suzuki 1962 Abe 196?) The late decrease in ERG amplitude can however not be due to removal of tonic

inhibition All the changes in the ERG might be due to retrograde alterations in the membrane properties of the elements generating the ERG A slow decrease in the membrane potential is compatible with an initial increase and a later decrease in the post synaptic propagated nervous activity in the retina Eccles Libet and Young (1958) found however, no decrease in membrane potential up to about 6 weeks after axotomy of the spinal motor neurones nor did they find any increase in the monosynaptic reflex discharge Their findings thus indirectly support the idea that the early increase in the ERG is due to an efferent control of retinal excitability

The efferents to the cochlea are known mainly to inhibit activity elicited by low intensity sound in the medium frequency range (Wiederhold 1967) Furthermore the efferent terminals are unevenly distributed in the organ of Corti (Ischii and Balogh 1968) Corresponding information on the control of retinal excitability is lacking but a dependence on stimulus parameters might explain the divergent results obtained by various authors (in the cat Jacobson and Suzuki 1962 and Brindley and Hamasaki 1962, in the rabbit the present study and Abe 1962)

An alternative explanation for the initial increase in ERG amplitudes was proposed by Ogden (1968) This author interpreted the increase as due to an altered circulation in the denervated eye The main vessels that might be damaged on optic nerve section are the central retinal artery and vein which enter the optic nerve 2 mm behind the bulb However in the retina of the rabbit contrary to the monkey and the cat retina there are no capillaries from these vessels supplying the receptors and the nuclear layers All oxygen diffuses from the choroid Furthermore the rabbit has been shown to have a high anerobic glycolytic capacity (Noell 1959) Thus the *a* and *b* waves of the ERG are independent of the retinal circulation that originates from the central retinal artery in the optic nerve An altered circulation consequently is not a plausible explanation for the initial ERG increase following transection of the optic nerve

The cytological changes in the retina in the present study are slight as compared to earlier findings (van Buren 1963 Stone 1965 Horsten and Winkelman 1969) Several factors have however been found to influence the course of the retrograde changes in the cell body e g in motoneurones The age of the animal and to a great extent also the length of the axon between the lesion and the soma determine the fate of the cells (for review see Cragg 1970) It might be suggested that these factors are also crucial in the development of cellular degeneration in the retina

It can be proposed that the cytological changes found in the ganglion cell layer 22 months after the transection (see Fig 2) are related to the final decrease in ERG amplitudes recorded at the same time This relatively small but statistically significant decrease in ERG not found in the puppy 18 months after transection (Horsten and Winkelman 1969) might be the first functional sign of an incipient retrograde degeneration

A transsynaptic degeneration in the monkey retina was found in the inner nuclear layer 20 months after optic nerve section (van Buren 1963) The *b* wave is generated in this layer whereas the *a* wave is known to be a receptor potential (see e g Brown

1968) It would be reasonable to expect the *b* wave to be less than the *a* wave *if* the ERG had been recorded in van Buren s experiment In our study, as well as that on the puppy (Horsten and Winkelman 1969), neither signs of transsynaptic degenera tion nor selective changes in the ERG components were found It is possible however, that such changes would have developed if the animals had been allowed to live longer

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Acta physiol. scand 1971 82. 282—288

From the Department of Pharmacology, University of Goteborg, Sweden

The Effect of Protriptyline on Uptake and Retention of dl-³H-Noradrenaline in Different Tissues of the Rat

By

OLLE ALMGREN and JAN JONASON

Received 16 December 1970

Abstract

ALMGREN, O and J JONASON *The effect of protriptyline on uptake and retention of dl-³H-noradrenaline in different tissues of the rat* Acta physiol scand 1971 *82* 282—288

The inhibition of ^{3}H noradrenaline (^{3}H NA) uptake in submaxillary glands spleens and hearts of the rat was studied after pretreatment of the animals with different doses of protriptyline (PTP) One µg/kg ^{3}H NA was administered intravenously 15 min after the intraperitoneal injection of PTP *The duration of the uptake blockade induced by 10 mg/kg PTP* was also studied To circumvent problems due to the blood flow, an *in vitro* technique was also used to compare the uptake and retention of ^{3}H NA in submaxillary glands and hearts It was found that the ^{3}H NA uptake of the heart *in vivo* normally exceeds several times that of the submaxillary glands while *in vitro* the uptake was directly correlated to the concentration of endogenous NA The data support the view that the greater blood flow through the heart is responsible for the major part of the difference found *in vivo* After PTP pretreatment the ... *vivo* ... the ... to ... *ter*

Exogenously administered noradrenaline (NA) is efficiently concentrated within the adrenergic neurons (Muscholl 1960 Whitby, Axelrod and Weil Malherbe 1961, Dengler Spiegel and Titus 1961) by a specialized uptake mechanism located at the level of the cell membrane, the membrane pump" (Hillarp and Malmfors 1964) This uptake mechanism can be inhibited by tricyclic antidepressive agents e g protriptyline (PTP) and desmethylimipramine (DMI) (Carlsson and Waldeck 1965) Recently it was reported by Maitre and Staehelin (1968) that DMI inhibited the uptake of i v injected ^{3}H NA much more efficiently in the heart than in the salivary glands of the rat The uptake of ^{3}H NA into the vas deferens was even augmented by DMI These findings prompted us to study the inhibitory effect of PTP on the ^{3}H-NA uptake *in vivo* in various organs of the rat The differences found in ^{3}H NA uptake both normally and after PTP treatment, was further analyzed with an *in vitro* technique In this way it is possible to circumvent the problems due to differences in regional blood flow

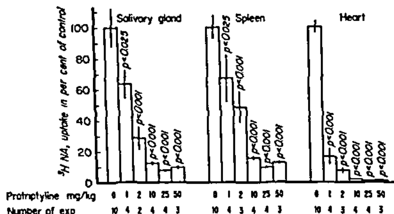


Fig 1 The effect of different doses of protriptyline on the ^{3}H noradrenaline content in the s

heart In the salivary glands and in the spleen the uptake after this dose of PTP was about 15 per cent of that found in untreated animals This difference in inhibition of uptake between heart and salivary glands or spleen was also found to be statistically significant ($P<0\ 001$) The highest dose of PTP investigated (50 mg/kg) did not induce any further reduction of the uptake capacity in the organs

The duration of the uptake blocking effect of PTP (10 mg/kg) is illustrated in Fig 2 The maximal blocking effect was short lasting in the spleen and the salivary glands Furthermore, in these organs the uptake capacity of ^{3}H NA had returned to normal within 12 hrs In the heart on the other hand the uptake of ^{3}H NA remained on a very low level for at least 3 hrs The uptake capacity of the heart significantly differed from that of the salivary glands or the spleen between 45 min and 6 5 hrs after PTP ($P<0\ 001$) 12 hrs after the injections of PTP there was still a reduced uptake of ^{3}H NA in the heart ($P<0\ 05$)

The endogenous NA content of the submaxillary glands was found to be $1\ 21 \pm 0\ 095$ μg/g (n=6) and of the heart $0\ 99 + 0\ 120$ μg/g (n=6) The difference is not significant ($P>0\ 10$ calculated with Student s t test)

Effect of PTP on the uptake and retention of ^{3}H-NA in vitro

From Table I it can be seen that without treatment the salivary gland slices tended to retain slightly more ^{3}H NA than the heart slices ($P<0\ 10$) Addition of 5 μg PTP to the incubation fluid resulted in highly significant reductions in the amount of retained amine in both the salivary glands and the hearts With this treatment only

Material and Methods

In vivo experiments

Male Sprague Dawley rats weighing about 200 g were kept in an environmental temperature of 29° C PTP in different doses was given intraperitoneally (i p) 15 min before an i v injection of 1 μg/kg dl noradrenaline 7 ^{3}H (specific activity 9 7 C/mM New England Nuclear Chemicals) 30 min after the administration of ^{3}H NA the rats were killed by a blow on the head. The heart spleen and submaxillary plus sublingual glands were immediately taken out and weigh
10 ml of
chromatog
and Wald
tritium content was measured in a Packard Tri-Carb Liquid Scintillation Counter

In another experiment 10 mg/kg PTP was given to rats of the same size and under the same conditions as above At different time intervals after the PTP injection 1 μg/kg *dl* ^{3}H NA was given i v 30 min after the ^{3}H NA administration the rats were sacrificed and the organs taken out and treated as described above In some untreated rats of the same size the endogenous NA content of the heart and the submaxillary plus sublingual glands was measured according to the trihydroxy indole method described by Bertler Carlsson and Rosengren (1958)

In vitro experiments

Adult male Sprague Dawley rats weighing about 200 g were used Some of the animals were pretreated with 10 mg/kg PTP by an i p injection 30 min prior to death The rats were sacrificed by a blow on the head. The hearts and the submaxillary plus sublingual glands were removed, weighed and immediately sliced. The hearts were divided into two pieces before
s obtained from one
each half in a single
10 min at 37° C in
e experiments 5 μg
period 500×10^{12}
moles of *dl* ^{3}H NA (10^{-6} curies) was added and the flasks were incubated an additional 20 min Control samples were prepared by addition of 2 ml 2 N HCl to the flasks before the pre incubation period

After 20 min of incubation the slices were blotted with filter paper and incubated an add 10 min in a substrate free medium. The slices were then blotted again and added to 2 ml of 2 N HCl. The samples were homogenized and centrifuged and the sediments then re extracted with an additional 2 ml of 2 N HCl. After the second homogenization and centrifuga t on the combined supernatants were filtered The volume of the filtrates was reduced to dry ness in a freeze-drier liquid scintillation fluid was added and the total radio-activity determined in a Packard Tri-Carb Liquid Scintillation Counter The values for retention of NA were calculated by correcting for efficiency and specific activity of the substrate and are presented as moles×10^{-12} per g tissue If not otherwise stated the P values were calculated from t tests after an analysis of variance which was performed after logarithmic transformat on of the values

Results

Effect of PTP on the uptake of ^{3}H NA in vivo

The content of ^{3}H NA 30 min after the injection of the amine into untreated rats varied considerably between different organs In the salivary (*i e* submaxillary plus sublingual) glands 1 13±0 141 ng/g was found in the spleen 0 38±0 079 ng/g while in the heart the content of ^{3}H NA amounted to 3 91±0 151 ng/g

The effect of PTP upon the uptake of ^{3}H NA is presented in Fig 1 PTP in a dose of 1 mg/kg reduced the uptake of ^{3}H NA in the heart to less than 20 per cent of normal while the corresponding reduction in the salivary glands and in the spleen was only about 65 per cent. This difference in uptake capacity is significant at P<0 001 10 mg/kg PTP nearly completely abolished the ^{3}H NA uptake in the

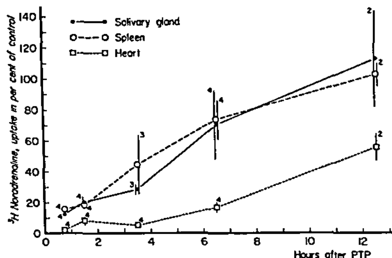


Fig 2 The ^{3}H noradrenaline uptake in the salivary gland, spleen and heart of the rat at different time intervals after the i p administration of protriptyline (10 mg/kg) ^{3}H nor- [illegible] is given in Fig 1

TABLE I Retention of ^{3}H noradrenaline in slices of rat salivary glands and hearts.

Slices of salivary glands and hearts were incubated for 20 min with 500×10^{12} moles of *dl*-^{3}H noradrenaline (10^{-6} curies) in 5 ml Krebs Henseleit solution In some experiments [illegible] (control values subtracted) n represents the number of experiments

Treatment		moles × 10^{12} per g tissue	
		Salivary gland	Heart
No treatment	Mean	196 9	160 6
	S E M	13 2	10 8
	n	35	27
Protriptyline 1 µg/ml *in vitro*	Mean	69 2[1]	11 7[1]
	S E M	5 09	0 82
	n	17	11
Protriptyline 10 mg/kg i p 30 min prior to death	Mean	80 8[1]	21 1[1]
	S E M	6 51	4 65
	n	16	7

[1] significantly different from normal values at $P < 0.001$.

about 7 per cent of the normal retention ability was detectable in the hearts whereas the corresponding figure of the salivary glands was 35 per cent This difference in the reduction of amine retention between the hearts and the salivary glands is also highly significant ($P<0.001$) When the rats were injected with 10 mg/kg PTP i p 30 min prior to death, the retention ability in the hearts and salivary glands was reduced to 13 and 41 per cent of normal respectively Also this difference in the NA retention ability between hearts and salivary glands after PTP administered *in vivo* is highly significant ($P<0.001$)

Discussion

It is known that the *in vivo* uptake of exogenously administered labelled NA varies between different organs (Whitby Axelrod and Weil Malherbe 1961) Also in the present study differences in the uptake of ^{3}H NA was observed between the salivary glands spleen and heart of the rat Thus the uptake of ^{3}H NA in the heart was about 4 times greater than in the submaxillary gland and the uptake of the amine in the spleen was even lower than in this gland The density of adrenergic nerve terminals in the organ is one factor that determines the extent of uptake (Kopin Gordon and Horst 1965) another factor is the magnitude of the regional blood flow (Wurtman *et al* 1964) The density of adrenergic nerve terminals is roughly reflected by the endogenous NA content In our material the NA content of the submaxillary gland tended to be somewhat greater than that of the heart (submaxillary gland/heart 1 2/1) This is also the case in e g the material of Maitre and Staehelin (1968) It thus appears that the density of the adrenergic nerve terminals is if anything slightly greater in the submaxillary gland than in the heart Consequently this factor cannot account for the difference in ^{3}H NA uptake between the heart and the submaxillary gland

Kopin Gordon and Horst (1965) have investigated the relative blood flow through different organs of the rat using the method of ^{42}K distribution They found that the relative blood flow through the heart was greater than that of the salivary glands Differences in blood flow might thus be the main factor accounting for the differences in NA uptake observed between the heart and the salivary glands in the present study Further support for this view is given in Table I In this part of the study an *in vitro* technique with tissue slices was used in order to escape difficulties due to the circulation When tissues slices from the two organs were incubated with ^{3}H NA the ratio between the amounts retained in the salivary gland and the heart was found to be 1 2/1 which exactly corresponds to the endogenous NA concentration in the two organs It is thus very likely that the much greater uptake of ^{3}H NA into the heart *in vivo* is mainly due to a greater blood flow The hypothesis of a specific affinity of the heart for the sympathetic transmitter proposed by Raab and Gigee (1955) is not supported by these data

The uptake of ^{3}H NA is inhibited at the level of the cell membrane as previously mentioned by different tricyclic thymoleptic agents PTP which was used in the

present study, inhibited the uptake of ^{3}H-NA much more efficiently in the heart than in the submaxillary gland or the spleen at all doses studied (Fig 1) Furthermore, the duration of this blockade was also considerably longer in the heart than in the other organs (Fig 2) This is in good agreement with the results of Maitre and Staehelin (1968) using DMI

Several possible explanations could be offered for the fact that the degree of uptake inhibition induced by the tricyclic thymoleptics varies between different organs The distribution of the substance might be affected by the magnitude of the blood flow through the organ Also, the substance itself might influence the distribution of ^{3}H NA by changing this blood flow Furthermore, there could be a true difference in sensitivity to agents of this group between different organs Extraneuronal binding has been proposed to play a certain role in amine uptake of the salivary glands (Fischer *et al* 1965, Almgren Anden and Waldeck 1965, Hamberger, Norberg and Olson 1967) Such a binding could possibly influence the results as was also proposed by Maître and Staehelin (1968) The uptake inhibiting effect of the tricyclic thymoleptics would in that case, be partially masked since these agents probably are effective only at the nerve cell membrane (see Jonason 1969)

To eliminate the possible circulatory influence on the difference in PTP effect, the uptake of ^{3}H NA was also studied *in vitro* In these experiments it was clearly shown that PTP, either when present in the incubation medium or when given i p to the animals before killing induced a greater inhibition of the ^{3}H NA uptake in the hearts than in the submaxillary glands Thus, even though circulatory factors may be of some importance *in vivo*, they do not entirely explain the difference in the PTP effect between the heart and the submaxillary gland The most probable explanation is at present that the difference found is partly due to an extraneuronal accumulation of ^{3}H NA in the salivary glands, that is not influenced by PTP However, from the present study the possibility can not be ruled out that there exists a true difference in sensitivity to PTP between the adrenergic nerves of the heart and the submaxillary gland Experiments are being conducted to settle this question

The research reported in this study has been sponsored by ... Council (No B 71 14X 2862 0? and B 71 14X 155 ... tina Lundgrens Vetenskapsfond We are sincerely g... of Mrs Gunilla Jonason We are also indebted to R... expert assistance in the preparation of the figures

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From the Coagulation Research Laboratory and the Surgical Research Laboratory, Thoracic Clinic, Karolinska Institutet, Stockholm, Sweden

Fibrinogen Degradation Products in Experimental Intravascular Coagulation

Gel filtration and immunological studies

By

ERIC ZETTERQVIST

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Abstract

ZETTERQVIST, E. *Fibrinogen degradation products in experimental intravascular coagulation.* Acta physiol. scand. 1971. 82. 289—299.

Dogs injected with ^{125}I fibrinogen were given 1) a thrombin infusion during 1 hr and 2) simultaneous infusions of epsilon aminocaproic acid (EACA) and thrombin, respectively ...

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Increasing evidence has been obtained that consumption of coagulation factors is accompanied by fibrinolysis in intravascular coagulation. In several experimental studies using procoagulants the mechanisms involved have been investigated (Kowalski *et al.* 1965, Nordström and Zetterqvist 1966, Girolami *et al.* 1966, Leandoer and Nilehn 1968, Teger-Nilsson 1969). For the evaluation of the total effects obtained in such experiments and also with respect to explanation and diagnosis in clinical intravascular coagulation, the action of two different proteolytic mechanisms must be taken into account.

As to the initial and limited proteolysis brought about by thrombin, through which fibrinopeptides A and B are released from the fibrinogen molecule, only a few studies have been made during the conditions of experimental intravascular coagulation. After thrombin infusion in dogs, peptides with the behaviour of fibrinopeptide A have been isolated and their potential diagnostic significance has been discus-

(Teger Nilsson 1969) Concerning fibrinopeptide B it has been demonstrated that in ^{131}I-labelling of dog fibrinogen this peptide is tagged with up to 9 % of the total radioactivity in the native protein (Nordstrom *et al* 1966, Blomback and Zetter qvist 1968) Regoeczi and Walton (1967) found about 11 % of the total radioactivity in the supernatant after thrombin coagulation of labelled dog fibrinogen These findings are of relevance for the evaluation in studies on the elimination of labelled fibrinogen

With respect to products obtained as result of advanced proteolysis of fibrinogen and fibrin by plasmin several studies have been made, in which different components have been described as to their molecular size, immunological behaviour, and their influence upon normal blood coagulation (Niewiarowski and Kowalski 1957, Stor morken 1957, Triantaphyllopoulos 1957 and 1965, Nussenzweig and Seligmann 1960, Fletcher *et al* 1962, Nilehn and Robertson 1965, Fisher *et al* 1967, Nilehn 1967, and others) Leandoer and Niléhn (1969) in studies on the effect of thrombin in dogs found 'split' products of fibrinogen or fibrin mainly in the "19 S" peak, *i e* corre sponding to the macroglobulins One most valuable contribution to the under standing of pathological fibrinogen degradation seems to be the study of Lipinski *et al* (1967), showing *in vitro* that fibrinogen fibrinogen fibrin intermediates and fibrinogen degradation products (FDP) may form soluble complexes, unclottable by thrombin

The present study was made in order to try to separate by gel filtration the fibrinogen degradation products obtained after intravenous infusion of thrombin and characterize these products with respect to their immunological behaviour and electrophoretic mobility in relation to fibrinopeptides A and B from dog blood

Material

9 *dogs* 15—20 kg were used in the experiments
Dog fibrinogen and ^{12} *I dog fibrinogen* were obtained as described earlier (Nordstrom and Zetterqvist 1968)
Thrombin was the preparation Thrombin Topical® by Parke Davis and Co
Sepharose 4 B and Sephadex G 25 were the products of Pharmacia Upsala Sweden
Epsilon aminocaproic acid (EACA) was the product Epsikapron® of KABI Stockholm Sweden
Antiserum Rabbit anti dog fibrinogen serum was obtained after 2 subcutaneous injections
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Methods

Thrombin infusion
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Gel filtration

1 For filtration of plasma *Sepharose 4 B* columns (5 0 cm² × 40 5 cm) were used, equilibrated with a Na phosphate buffer, pH 7 0 (0 05 M Na_2HPO_4, 0 05 M NaH_2PO_4, 0 5 M NaCl) The flow was around 12 ml/hr The eluate was collected in fractions of 2—3 ml. The void

Radioactivity counting

Radioactivity of the effluent of the gel filtration columns was measured in a liquid scintillation spectrometer (Beckman CPM 200). To 0 5 ml of sample from each tube were added 1 5 ml of 10 % HAc and 10 ml of a scintillation liquid (naphtalene 100 g, 2 5-diphenyloxazole 5 g, dioxane up to 1000 ml)

Electrophoresis

Germany) was used

Immunological techniques

Immunoelectrophoresis was performed by a conventional method (Scheidegger 1955) on 1 *serum* obtained during the thrombin experiments at times as illustrated (Fig 1) and on 2 *samples of the eluate after gel filtration on Sepharose 4 B* The serum and antiserum were undiluted, the eluate fractions were freeze-dried and dissolved in water The samples were run for 2 hrs at 70 V, 12 mA and then allowed to diffuse against the antiserum for 48 hrs The plates were then photographed

Immunodiffusion was performed according to Ouchterlony (1949)

Results

Thrombin infusion

The results of the experiments in the 2 dogs, given thrombin and thrombin together with EACA, respectively are illustrated in Fig 1 The radioactivity curves had the appearance as in earlier experiments (Nordstrom and Zetterqvist 1968). It can be

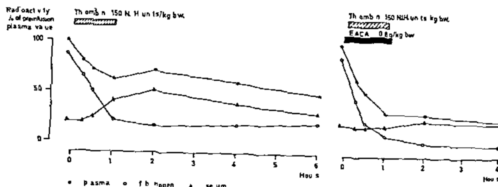


Fig 1 Infusion of *thrombin* (dog no 583) and *EACA together with thrombin* (dog no 581) Plasma, fibrinogen and serum radioactivity

noticed that the radioactivity in serum before the thrombin infusions was around 20 % of that in plasma (Table) The mean value from determinations in 9 dogs was 25 % During the thrombin infusion, the serum radioactivity in the dog given thrombin alone rose rapidly and from 1 hr after the start, the serum curve ran paralell with that of plasma In the dog, to which also EACA was administered, there was late in the experiment a small increase in serum radioactivity At 2 hrs after the start

TABLE

Dog nr	Radioactivity distribution in blood — %			Fibrinogen concentration		Radioactivity distribution in eluate after Sepharose 4 B filration — % of initial plasma value			Radioactivity recovered after Sepharose 4 B filtration — % of input
	plasma	fibrinogen	serum	g/100 ml	% of ml	peak I initial	peak II	IR	
583									
before experiment	100	87	21	0 46	100	64	14	17	98
at 2 hrs after start of thrombin infusion	71	16	52	0 11	24	30	21	17	79
581									
before experiment	100	87	21	0 37	100	61	14	17	95
at 2 hrs after start of thrombin infusion + EACA	32	4	26	0 04	11	3	12	5	

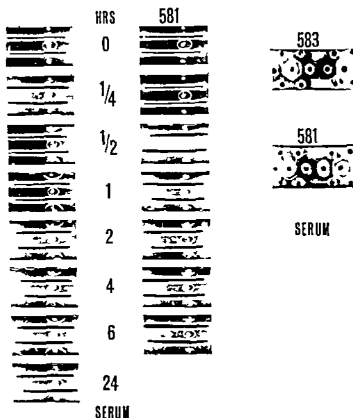


Fig 2 Immunoelectrophoresis (left) and immunodiffusion (upper right) on consecutive serum samples in connection with infusion of *thrombin* (dog no 583) and of *EACA together with thrombin* (dog no 581)

of the thrombin infusion, the fibrinogen radioactivity in the dog given thrombin + EACA had fallen to 4 % whereas in the dog given only thrombin the corresponding activity at the same time was still at 16 % of the initial plasma value (Table)

The results obtained with *immunoelectrophoresis* and *immunodiffusion* on serum obtained from the dogs before during and after the experiments with thrombin and thrombin + EACA respectively are illustrated in Fig 2 In both dogs there were in immunoelectrophoresis faint precipitates already before the start of thrombin infusion In the dog given only thrombin (no 583) strong arches were obtained in the samples taken from 1 to 6 hrs after the start On the following day there was still a faint precipitate In the dog given EACA in addition to thrombin there were precipitates weaker and appearing later than in the dog given only thrombin The results obtained with immunodiffusion on the same samples corresponded well Thus, there seems to be after thrombin infusions products appearing with the antigenic determinants of fibrinogen

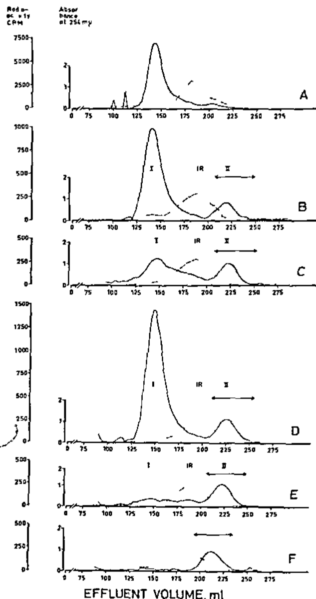


Fig 3 Separation of fibrinogen and degradation products on Sepharose 4 B A calibration of column B D plasma before and C, E plasma after infusions of thrombin and EACA + thrombin, F clot supernatant after thrombin coagulation of plasma before thrombin infusion ←→ fractions filtered on Sephadex G-25

Gel filtration through Sepharose 4 B

To get an information on the molecular sizes and immunological behaviour of the degradation products appearing before and likely to be found in increasing amounts after the thrombin infusions, gel filtration through a Sepharose column of different plasma samples was performed (Fig 3)

Tracing of the protein content as light absorbance at 254 mu, in all filtrations showed that most of the protein material was eluted at effluent volumes between 160 and 210 ml The pattern of the curve appeared to be the same in all experiments (Fig 3)

A *Calibration of column*

…) + 2 7 ml of normal dog … volume of 145—150 ml … bviously representing the … at an effluent volume of

B D and F *Filtration of plasma and serum obtained before thrombin infusions*

After filtration of plasma (2 6 ml 20—70 μC × 10^{-3}), obtained at complete distribution and steady state catabolism after injection of ^{125}I fibrinogen and before the thrombin experiments in both dogs used (no 583 581), 2 distinct radioactivity peaks were demonstrated (B D) Of …

… therefore consisted of material with lower molecular weight than that of fibrinogen In front of the latter only small amounts of radioactive material (4 %) was eluted

C *Filtration of plasma obtained after thrombin infusion*

Filtration was performed of plasma (2 6 ml, 20—70 μC × 10^{-3}) from a sample drawn at 2 hrs after the start of the thrombin infusion (C), *i e* at the point of estimated maximum for secondary fibrinolysis (Fig 1) The radioactivity distribution was compared with that in plasma before thrombin infusion, the radioactivity content in peaks I and II and in IR calculated as …

… weight products The IR radioactivity was not changed The sum of the contents in peak II and IR together with the estimated nonclottable radioactivity in peak I, *i e* around 44 % corresponded fairly well with the simultaneously occurring radioactivity in serum of 52 % (Table)

E *Filtration of plasma obtained after simultaneous infusions of thrombin and EACA*

…

As mentioned above *immunological studies* on the serum samples obtained during the experiments demonstrated the appearance of products with the antigenic determinants of fibrinogen To try to find out if these determinants were confined to degradation products separated by the Sepharose column immunoelectrophoresis was also performed on consecutive samples after Sepharose 4 B filtration in all studies Eight to nine fractions from every filtration experiment were tested Material with

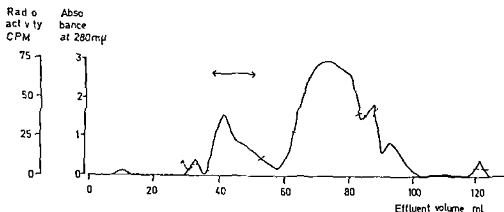


Fig 4 Separation of low molecular weight products on Sephadex G 25 Peak II obtained after Sepharose filtration of clotsupernatant

the antigenic determinants of fibrinogen was recovered in samples from effluent volumes between 140 and 200 ml There were positive tests over all the region mentioned in the plasma samples obtained before thrombin infusion (B D) as also in that obtained at 2 hrs after thrombin infusion without EACA (C) However in e dog given thrombin +EACA (E) only material within the IR gave faint precipitates Thus there are degradation products of fibrinogen with the antigenic determinants of the protein

Gel filtration on Sephadex G 25 and thin layer electrophoresis

From the Sepharose filtration pattern it seemed possible that part of the radioactivity recovered in peak II, being eluted at the total volume might consist of relatively low molecular weight fibrinogen degradation products It was desirable to get a better information on the molecular size of the actual products and particularly to see if fibrinopeptide B could be demonstrated The incorporation of iodine into this fibrinopeptide would facilitate its demonstration

As the molecular weight of the dog fibrinopeptides can be estimated to around 2000 (Blomback *et al* 1966) Sephadex G 25 was chosen for further separation studies The eluates from effluent volumes between about 190 and 250 ml from the Sepharose filtrations (Fig 3) were pooled freeze dried and after dissolution in water put on the columns

After Sephadex filtration radioactive material was found mainly in material eluted between effluent volumes from 65 to 95 ml Besides there was in peak II material from clot supernatant (F) a radioactivity peak corresponding to effluent *volumes* around 35 to 55 ml (Fig 4) Obviously the major part of the radioactivity in peak *II* was confined to low molecular weight products

Tracing of the protein content as light absorbance at 280 mμ showed small amounts at an effluent volume of 40 ml and the major part of protein at affluent volumes between 50 and 100 ml The pattern of the curve appeared to be essentially the same in all experiments

At least fibrinopeptide B being radioactive would be expected to appear in the material corresponding to the radioactivity peak first eluted Therefore with guidance by the results of radioactivity tracing on the eluate of the peak II material from the clot supernatant (Fig 4) the eluate fractions obtained between effluent volumes from around 40 to 50 ml in all Sephadex filtrations were pooled After freeze drying the material dissolved in water was run in thin layer electrophoresis together with reference fibrinopeptides A and B In no sample there could be demonstrated material identifying with fibrinopeptide B There was however a ninhydrin positive spot with the mobility of fibrinopeptide A in clot supernatant but also in plasma obtained before the thrombin infusions The significance of the latter observation is doubtful

Discussion

As to the characterization of fibrinogen split products obtained in clinical pathological fibrinolysis the group of Fletcher *et al* (196? 1966) including also Fisher (1967) have demonstrated three classes of fibrinogen products occurring in varying proportions depending on the degree of the abnormal plasma proteolysis In mild clinical fibrinolysis there seems to be a predominance of high molecular weight products whereas in the advanced state a wide variety of fibrinogen degradation products can be demonstrated (Fisher *et al* 1967 Nilehn 1967) Concerning the effects upon fibrinogen after intravenous thrombin infusions in dogs evidence has been obtained for the occurrence of fibrinogen split products (Kowalski *et al* 1965 Nordstrom and Zetterqvist 1966) Leandoer and Nilehn (1969) also studying the effects of thrombin demonstrated by gel filtration split products of high molecular weight

In this study there was already before the thrombin infusions in serum obtained after spontaneous clotting in glass tubes containing EACA around 20 % of the radioactivity in plasma The same finding was made by Leandoer and Nilehn (1969) In contrast Kowalski *et al* (1965) in corresponding studies found only a few per cent of the radioactivity in serum They however noted this amount at less than 2 hrs after the injection of ^{131}I fibrinogen *i e* before complete distribution and steady state catabolism had been reached An amount of 20 % is somewhat higher than what would be expected from the difference of 13 % between the plasma activity and that of fibrinogen (determined as fibrin) This could be explained by co-polymerization of high molecular weight degradation products during fibrin formation in thrombin coagulation (Alkjaersig *et al* 196?) Such an interpretation is also corroborated by the finding that the degradation products demonstrated in plasma before thrombin infusion could not be found to any appreciable extent after coagulation of a corresponding plasma sample with thrombin

There was also demonstrated in serum before thrombin infusion the presence of material with the antigenic determinants of fibrinogen That this is represented by fibrinogen degradation products is probable as in the filtration studies products penetrating the gel more than fibrinogen were found These also contained the antigenic determinants of fibrinogen The presence of these products despite the fact that no increased plasma fibrinolytic activity has been demonstrated in the blood before and not even after thrombin infusions (Nordstrom and Zetterqvist 1966 1968) indicates that the degradation occurs in compartments separated from the circulating blood

The present study shows that in dogs after thrombin infusion and obviously even before there is a wide variety of fibrinogen degradation products With respect to molecular weight the range seems to be from products with the same elution as fibrinogen on Sepharose 4 B down to those retained by Sephadex G 25 i e between molecular weights 340 000 and 5 000—1 000 Such a wide variation for fibrinogen degradation products has been demonstrated in clinical intravascular coagulation (Fletcher *et al* 1966 Fisher *et al* 1967 Nilehn 1967) The high molecular weight products (peak I IR) contained the antigenic determinants of fibrinogen whereas peak II did not In peak I there seemed to be besides fibrinogen also non clottable high molecular weight degradation products as the radioactivity of the fibrinogen peak after thrombin infusion was larger than would be expected from the corresponding fibrinogen concentration in plasma

No radioactive material with larger molecular weight than that of fibrinogen was observed in the Sepharose chromatograms of plasma obtained after the thrombin infusions This would indicate that complexes of fibrinogen or fibrin and degradation products as demonstrated in vitro by Lipinski *et al* (1967) do not appear in connection with thrombin infusion to dogs

The results obtained in this study are in favour of the view that fibrinogen degradation products increase after thrombin infusions and thus seem to be indicative of a secondary fibrinolysis This conclusion is further stressed by the pronounced inhibition by EACA of the appearance of degradation products

Recently Teger Nilsson (1969) has found that fibrinopeptides split off from the fibrinogen molecule during coagulation are rapidly degraded in serum As the fibrinopeptide B is partially labelled during iodination of fibrinogen (Nordstrom *et al* 1966 Blomback and Zetterqvist 1969) an attempt was made to isolate this peptide from plasma of defibrinated dogs No evidence for the occurrence of fibrinopeptide B or derivatives of it could however be obtained This might indicate that fibrinopeptide B is released at a slow rate during coagulation or rapidly degraded to smaller fragments escaping detection with the methods used for isolation In electrophoresis there was a ninhydrin positive spot with the mobility of fibrinopeptide A in clot supernatant but also in plasma obtained before thrombin infusion This finding therefore is obscure

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From the Department of Medical Microbiology and the Department of Pharmacology, University of Lund, Sweden

Endotoxin-induced Suppression of Rabbit Kidney DOPA Decarboxylase Activity

By

HANS FRITZ and ROLF HÅKANSON

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Abstract

FRITZ, H and R HÅKANSON *Endotoxin-induced suppression of rabbit kidney DOPA decarboxylase activity* Acta physiol scand 1971 82 300—309

The kinetic properties of the histamine forming enzyme in the cortex of the rabbit kidney were found to be identical with those of DOPA decarboxylase There was no evidence of "specific" histidine decarboxylase in the rabbit kidney cortex Following i v injection of endotoxin, reduced DOPA decarboxylase activities were demonstrated after 3 and 6 hrs, after 24 hrs low as well as conspicuously high enzyme activities were observed

DOPA decarboxylase is of importance for the formation of, for instance, catechol amines in the central and sympathetic nervous systems and for the formation of 5-hydroxytryptamine in the bowel (for references see Håkanson 1970) Probably, the enzyme is capable also of decarboxylating *L*-histidine (Rosengren 1960, Lovenberg *et al* 1962), although the physiological significance of the histamine-forming capacity of DOPA decarboxylase has been questioned (Ganrot *et al* 1961) Apart from DOPA decarboxylase, another more specific histamine-forming enzyme has been demonstrated in some mammalian tissues This enzyme is referred to as "specific" histidine decarboxylase (Ganrot *et al* 1961, Burkhalter 1962 Håkanson 1963 1967 a and b) High activities of both DOPA decarboxylase (Schales and Schales 1949, Clark *et al* 1954, Rosengren 1960, Lovenberg *et al* 1962) and "specific" histidine decarboxylase (Rosengren 1966, Håkanson 1967 a) have been demonstrated in the renal cortex of some species

Stressful stimuli, including the administration of bacterial endotoxins, activate the histamine-forming capacity of murine muscle, skin and renal cortex (Schayer and Ganley 1959, Schayer 1960, 1962) In the mouse kidney, endotoxins cause a great increase in the histamine forming capacity, conceivably as a result of increased amounts of "specific" histidine decarboxylase In the rabbit kidney, however, the

histamine forming capacity is reduced after endotoxin administration (Schayer *et al* 1963). One explanation of this discrepancy may be that the histamine-forming enzyme in the rabbit kidney is different from that in the mouse kidney (Fritz and Håkanson 1969).

The aims of the present investigation were to identify the histamine-forming enzyme of the rabbit kidney and to analyze the response of this enzyme to endotoxin administration

Materials and methods

Rabbits delivered from local breeders and weighing 1 6—4 9 kg (mean 3 3 kg), they were fed pellets and water

Endotoxins from *Salmonella abortus equi* (Difco 3127) and *Escherichia coli* (O 111 B4, Difco 3122) are commercial products prepared according to Westphal (Westphal *et al* 1952, Westphal *et al* 1958) The endotoxins were dissolved in distilled water to a concentration of 100 or 200 μg/ml

One ml of the endotoxin solution was injected intravenously into an ear vein Before the injection a blood sample was collected The rabbits were bled to death by cardiac puncture

within 5 min

The kidney content of DOPA decarboxylase was assayed according to Håkanson (1966) within 5 days after death of the animal The assay was performed with material from one kidney from each animal 100 mg (wet weight) of tissue from the kidney cortex was

cortex was expressed as counts/min per 10 mg of tissue The standard deviation at the repetition of a single measurement was ±11 per cent of the value obtained

For the study of the kinetic properties of the histamine forming enzyme the kidneys were removed and taken for assay immediately after death An enzyme extract was prepared by

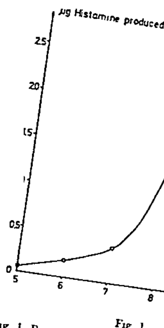


Fig 1

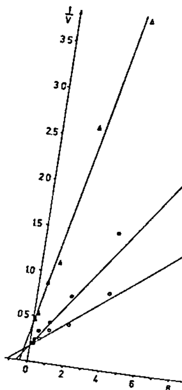


Fig 2

Fig 1 Rate of histamine formation (µg/hour) at various pH Substrate concentration
Fig 2 Double reciprocal plots of initial reaction velocities (v = µg histamine pe various histidine concentration ((S) = molarity), O—O, pH 9, ●—● pH 8, ∠—∠

Results

Kinetics of histamine formation catalyzed by rabbit kidney extract

The major portion of the histamine-forming enzyme of the phosphate buffer ex was recovered in the fraction precipitated by ammonium sulfate at betweer and 47 per cent salt saturation The pH optimum for histamine formation as c lyzed by this semi-purified preparation of rabbit kidney enzyme was close t (Fig 1) The pH optimum was the same with both 10^{-2} M and 10^{-3} M histidi The Michaelis-Menten constant was calculated from Lineweaver-Burk plots rela ing initial reaction velocities to the substrate concentration (Fig 2) and w $2\,2 \times 10^{-2}$ at pH 7, $1\,7 \times 10^{-2}$ at pH 8 and $0\,9 \times 10^{-2}$ at pH 9

Effects of endotoxin on rabbit kidney DOPA decarboxylase activity

Control experiments

The DOPA decarboxylase activity in the kidney cortex of 9 of the 10 control rabbits was 1400 ± 135 (mean ± S E M), range 974—1854 There was no correlation between body weight and DOPA decarboxylase activity The sera from one control

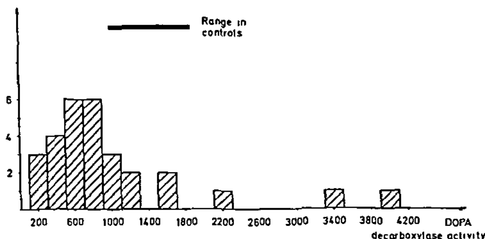


Fig. 3 DOPA decarboxylase activity of kidney cortex extract Total endotoxin treated material

rabbit without any obvious signs of disease proved to be cytotoxic both from the sample taken before the injection of distilled water and from a sample collected at cardiac puncture This rabbit was therefore excluded in the above calculation of DOPA decarboxylase activity in the control animals The DOPA decarboxylase activity in the kidney cortex of this rabbit was 596

Endotoxin treated rabbits

Total material 30 rabbits given various doses of endotoxin and killed at various time intervals after the injection The DOPA decarboxylase activity in the kidney cortex was 924 ± 123 The mean value of the endotoxin treated rabbits was significantly lower ($p < 0.02$) than that of the controls, 1400 Twenty five of the 30 endotoxin treated rabbits had a DOPA decarboxylase activity below the mean of the controls and 21 of these 25 had an activity below the range in the control experiments (Fig 3) Three of the endotoxin treated rabbits had an activity above the range found in the controls 2 of these were conspicuously different with a DOPA decarboxylase activity of 3309 and 3942 respectively These animals were injected with the 100 μg dose of endotoxin As illustrated in Fig 4 there was no correlation between body weight and DOPA decarboxylase activity after injection of 100 or 200 μg of endotoxin, per animal

Effect of endotoxin dose and of time interval between injection and death The experiments were completed with 24 rabbits injected with endotoxin from *S abortus equi* 16 of them were given 200 μg and 8 received 100 μg

The 8 rabbits injected with 100 μg were sacrificed after 24 hours Some of them were visibly ill at that time and in all the blood vessels of the ear were still constricted The mean value of DOPA decarboxylase activity in this series was 1538,

monkey kidney cells The cytoplasm of the cells became 'granulated with the nucleus appearing as a hollow space No correlation was found between the DOPA decarboxylase activity and the severity of the cytotoxic changes After injection of endotoxin only the serum from 1 of the 8 rabbits that was administered 200 μg of endotoxin from *S abortus equi* and sacrificed after 3 hrs failed to evoke characteristic cytotoxic changes The DOPA decarboxylase activity in the kidney cortex of this rabbit was 1562

Discussion

Except for a short communication (Fritz and Håkanson 1969) the effect of endotoxins on renal DOPA decarboxylase activity has not been described previously With regard to histamine forming enzymes however Schayer (1960 1962) demonstrated an increased activity in the kidney of the rat and mouse following different forms of stress *e g* burns intramuscular injection of catecholamines and intravenous injection of endotoxin Rabbit kidney however was different in that the histamine forming capacity was reduced after treatment with endotoxins (Schayer *et al* 1963)

The kinetic properties of semi purified preparations of the histamine forming enzyme of rabbit kidney revealed that the enzyme is identical with DOPA decarboxylase the pH optimum of the histamine forming reaction was found to be close to 9 and the Michaelis Menten constant was high at the pH values tested There was no evidence of specific histidine decarboxylase in the extract (For a discussion of the characteristic—and distinguishing—kinetic properties of these two histamine forming enzymes see Hakanson (1970))

Following injections of 200 μg of endotoxin we found a decrease of DOPA decarboxylase activity both after 3 and 6 hrs and a tendency to further decrease after 24 hrs The finding that endotoxins cause a decrease in the DOPA decarboxylase activity of the cortex of the rabbit kidney accords with previous observations of changes in the histamine forming capacity since it may now be assumed that the endotoxin induced suppression of the histamine forming capacity observed by Schayer *et al* (1963) reflects a reduced DOPA decarboxylase activity

After injection of 100 μg of endotoxin low as well as high values of DOPA decarboxylase were obtained after 24 hrs The explanation may be that with the 100 μg dose a turning point in the decrease of the enzyme activity occurs at about 24 hrs with a rapid reconstitution of the enzyme activity

The animals that received 2 injections each of endotoxin 24 hrs apart died spontaneously $1^1/_2$ hrs after the second injection In these animals the DOPA decarboxylase activity was of the same magnitude as that observed after injection of a single dose Following more frequently repeated injecti[illegible] toxin a drastically reduced DOPA decarboxylase activity, bel[illegible] 00 w[illegible] is finding may be related to a prolonged vasomotor r[illegible] te[illegible] of endotoxin (Zweifach *et al* 1956)

The pronounced pre- and post-capillary vasoconstriction following endotoxinaemia in the rabbit is dependent on elevated levels of circulating catecholamines (Rosenberg *et al* 1959, Heiffer *et al* 1960, Rosenberg *et al* 1961) combined with a markedly potentiated vascular response to epinephrine and norepinephrine (Tomas *et al* 1957) The mechanism resulting in kidney damage following endotoxinaemia is debatable (see *i a* Landy and Braun 1964) Endotoxin induced increased susceptibility of the small renal vessels to catecholamines is one suggested mechanism (Thomas 1956, Zweifach *et al* 1956, Fine 1964) Profound vasoconstriction *per se* may lead to renal damage (Penner and Bernheim 1940, Thal 1935) Vasodilators (Fine 1964) or sympathetic denervation of the kidney (Palmerio *et al* 1962) may protect against renal lesions following endotoxinaemia Accordingly, endotoxin induced changes in the rate of formation of catecholamines and/or histamine in the kidney may be critical Differences in the amine forming capacity may contribute to explaining the marked species variation in response to the endotoxins

The mouse kidney is known to contain both "specific" histidine decarboxylase and DOPA decarboxylase (Håkanson 1967 a), but probably, the endotoxin induced increase in the histamine forming capacity of the mouse kidney reflects an exclusive increase in the activity of the 'specific" histidine decarboxylase The mouse is up to 100 times more resistant to endotoxins than the rabbit This fact is conceivably due to the protecting effect of the 'specific" histidine decarboxylase since histamine opposes the vasoconstrictor action of the catecholamines (Schayer *et al* 1963, Spink *et al* 1963) The lack of 'specific" histidine decarboxylase in the rabbit kidney may, thus, predispose to ischemic renal lesions

A phenomenon of natural tolerance of endotoxin is at times frequently observed in experimental animals In the study of Schayer *et al* (1963) on endotoxin induced suppression of the histamine forming capacity of the rabbit kidney, about one third of the animals were reported to be tolerant of endotoxin In the present investigation we used the induced cytotoxicity of rabbit serum as an indicator of a response to the endotoxin administered Judging from the testing of serum cytotoxicity one of our rabbits was probably tolerant of the endotoxin injected The enzyme activity, 1562, of this animals was at variance with the low enzyme activities of the other animals in the group With this animal excluded a mean value of 766 instead of 866 is obtained for the 3 hours group in Fig 6 Another rabbit showed a spontaneously developed serum cytotoxicity possibly as a result of exposure to endotoxin from some infection Interestingly, this animal which was in the control group, had a DOPA decarboxylase activity of 596 — a level of activity otherwise found only among the endotoxin treated rabbits

The reduction in DOPA decarboxylase activity regularly observed in rabbit kidney cortex after injection of endotoxin may reflect a general degeneration of renal tissue, a release of substances depressing the DOPA decarboxylase activity or a more specific effect of the endotoxins on the DOPA decarboxylase-containing cell system The cellular localisation of renal DOPA decarboxylase is unknown. Because the capillary endothelium of the brain is a conspicuously rich source of DOPA

decarboxylase (Owman and Rosengren 1967), the possibility that the renal enzyme is located in the renal vessels may be considered The tentative hypothesis that a change in DOPA decarboxylase activity precedes an eventual kidney lesion can at present neither be confirmed nor refuted In contrast to what is found in rabbits that have died in a generalized Shwartzman reaction, the morphological evidence of renal damage following a single injection of endotoxin is sparse, although haem orrhages and necroses may be present in other internal organs (Gerber 1936) It is difficult to conceive how reduced renal DOPA decarboxylase could be respon sible for an endotoxin induced renal damage Increased activity, on the other hand could, possibly, be responsible for ischemic renal lesions if it is associated with an enhanced rate of formation of catecholamines It may be noted that at the time of a second injection of endotoxin 24 hrs after the first, high values of DOPA decarboxylase activity were demonstrated among the animals with a frequency that corresponds to that of inducible generalized Shwartzman reactions (Gerber 1936)

The similarities between rabbit and man in their response to the endotoxins have not been explained The unmodified generalized Shwartzman reaction can be in duced only in rabbits and in man (Hjort and Rapaport 1965) In a pilot study of surgical biopsy specimens of the human kidney, we have found the activity of human kidney DOPA decarboxylase to be of the same magnitude as that in the rabbit kidney Work is in progress to characterize the human kidney amino acid decarboxylase the similarities between rabbit and man in their response to the toxins may thereby be better understood

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decarboxylase (Owman and Rosengren 1967), the possibility that the renal enzyme is located in the renal vessels may be considered. The tentative hypothesis that a change in DOPA decarboxylase activity precedes an eventual kidney lesion can at present neither be confirmed nor refuted. In contrast to what is found in rabbits that have died in a generalized Shwartzman reaction, the morphological evidence of renal damage following a single injection of endotoxin is sparse, although haemorrhages and necroses may be present in other internal organs (Gerber 1936). It is difficult to conceive how reduced renal DOPA decarboxylase could be responsible for an endotoxin induced renal damage. Increased activity, on the other hand, could, possibly, be responsible for ischemic renal lesions if it is associated with an enhanced rate of formation of catecholamines. It may be noted that at the time of a second injection of endotoxin, 24 hrs after the first, high values of DOPA decarboxylase activity were demonstrated among the animals with a frequency that corresponds to that of inducible generalized Shwartzman reactions (Gerber 1936).

The similarities between rabbit and man in their response to the endotoxins have not been explained. The unmodified generalized Shwartzman reaction can be induced only in rabbits and in man (Hjort and Rapaport 1965). In a pilot study of surgical biopsy specimens of the human kidney, we have found the activity of human kidney DOPA decarboxylase to be of the same magnitude as that in the rabbit kidney. Work is in progress to characterize the human kidney amino acid decarboxylase: the similarities between rabbit and man in their response to the endotoxins may thereby be better understood.

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Since only a brief histochemical study on the ChE activity in human sympathetic ganglia (Cauna *et al* 1961) has been published the present work was designed to give some information on the activity and distribution of ChEs, monoamine oxidase (MAO) and catecholamines in human sympathetic ganglia.

Material and methods

Human sympathetic ganglia were removed during operations on patients treated at the Second Department of Surgery of the University of Helsinki The material was obtained from [illegible] (sympathectomia lumbalis [illegible] chloride 50 mg, atropine [illegible] esia, succinylcholine, thio- [illegible] ry operation, and in some [illegible] 15 min to 2 hr when the [illegible] rating surgeon within one [illegible] ganglia. In the ChE and [illegible] uring the operations were of sympathetic origin The whole ganglion was immediately frozen in liquid nitrogen and thereafter transferred to a plastic tube stoppered with a tight cork The ganglia were collected in the course of several weeks and stored in closed plastic tubes in a deep-freezer at −76° C for about 2 to 6 months Four further ganglia were obtained from accidentally killed men autopsied at the Department of Forensic Medicine The time interval between death and removal of the ganglia was never more than 24 hrs

Each ganglion was dissected out from its capsule and cut into several pieces in a cold room at −20° C, 2 pieces for histochemistry, 2 for fluorescence studies and one for quantitative enzyme assays

For quantitative enzymes assays the pieces of tissue were homogenized in 500 μl of a 0 1 [illegible] The assays were [illegible] method of Krajl [illegible] was seen The measurements were made on the Aminco-Bowman spetrophotofluorometer, using the optimal wavelengths for activation (325 nm, uncorrected instrumental value) and excitation (380 nm) [illegible]

Chem [illegible]

The [illegible] acetylc [illegible] (BuCh [illegible] gustin [illegible] methy [illegible] in a c [illegible] (Bayliss and Todrick 1956), and tetraisopropylpyrophosphoramide (iso-OMPA, L Light & Co Ltd, England) in a concentration of 10^{-5} M to inhibit non specific cholinesterase (ns ChE, E C 3 1 1 8) selectively (Aldridge 1953 Bayliss and Todrick 1956)

In most cases, the enzyme (50 μl) was preincubated with or without the specific inhibitor (5 μl) in 5 ml of 0 1 M KCl (CO_2-free, pH 8 0) for 30 min in a separate closed test tube Thereafter, the whole tube content was transferred to the reaction vessel and the substrate added [illegible] M serve [illegible] NaOH [illegible] were ad [illegible] slopes

Conditions in both MAO and ChE measurements were such as to give activities proportional to the time of incubation and amount of enzymes Protein was measured by the method of Lowry *et al* (1951), with bovine serum albumin as standard

The freeze-drying procedure and the formaldehyde-induced fluorescence method followed the principles of Eränkö (1967) The small pieces of tissue were freeze-dried at −40° C in a steel vessel with a thin layer of phosphorus pentoxide on the bottom for 4 days in a vacuum produced with a mechanical rotatory pump The vessel containing the specimens was then transferred to room temperature for 4 hr and the specimens allowed in vacuum to warm u[illegible] The specimens were then exposed to formaldehyde vapour derived from paraforma[illegible]

80° C for 90 min in a closed glass vessel. The humidity of the vessel was equilibrated at 60 %. After immersion in xylene the pieces of tissue were embedded in paraffin wax. Sections cut at 5 to 10 um were used for fluorescence microscopy.

For histochemistry the small pieces of ganglion were put into brain paste (0° C) spread on the tissue holder. Immediately after the frozen (−70° C) piece of tissue had been thrust into the paste, the holder was dropped into hexane cooled with dry ice, after which the holders were kept at −76° C until sectioned for histochemical study. Fresh sections were cut at 10 um on a cryostat at −25° C.

The distribution of the MAO activity was studied histochemically by the method of Glenner *et al.* (1957). No activity was found when the substrate was omitted or the sections had been incubated in the presence of 10^{-3} M iproniazide. The incubation times were 30 and 60 min.

Gomori's (1952) modification of Koelle's (1951) method was used for ChEs. The fresh sections were preincubated in the stock solution with or without inhibitor for 15 min at 37° C and thereafter incubated for 30 min or 2 hrs in the same medium into which the substrate acetyl-, propionyl- or butyrylthiocholine iodide respectively had been added. To differentiate between ChEs, the same inhibitors as in chemical determinations were used and at the same concentrations. Control sections were incubated without substrate. These controls always gave negative results.

Results

I. *Morphological Studies*

A. *Fluorescence studies*

The cytoplasm of most of the *large nerve cells* in the ganglion exhibited a greenish formaldehyde induced fluorescence. The fluorescent substance was evenly distributed in the cytoplasm of the nerve cells, the nuclear areas being non-fluorescent. The average intensity of the fluorescence in the nerve cells was weak, but in some cells the cytoplasm was moderately and in a few strongly fluorescent nerve cell cytoplasms were seen (Fig. 1—4). The intensity of the fluorescence faded rapidly during UV illumination and the bright greenish fluorescence of the cell bodies was totally abolished by treatment with sodium borohydride, which shows that it is due to a condensation product of catecholamine and formaldehyde (Corrodi *et al.* 1964). The cytoplasm of some of the large nerve cells exhibited no formaldehyde induced fluorescence. Closely adjacent to the cell body of the large nerve cells, strongly greenish fluorescent areas were occasionally seen, often having a more granular appearance than the cytoplasm of the nerve cell bodies. The width of these moderately fluorescent areas correspond to about 1/4 to 1/3 of the diameter of the nerve cell body and only an obscure impression of the possibly nonfluorescent nuclear area was found (Fig. 3). The fluorescent areas are probably small intensely fluorescent cells described in the rat superior cervical ganglion by Eränkö and Härkönen (1965) and Eränkö and Eränkö (1971). The *satellite cells* were non-fluorescent.

Some *small nerve cells* of the sympathetic tissue exhibited a greenish fluorescence identical to that seen in the large nerve cells. Small fluorescent nerve cells of this kind were usually sparse but sometimes large clusters of them were seen separated from the surrounding ganglion tissue by a thin connective tissue like sheath (Fig. 4). The over all fluorescence of the cytoplasm in the cells of these clusters was weak or occasionally moderate.

There were numerous delicate nerve fibres or bundles of them in the ganglion,



Fig 1—4 Formaldehyde induced fluorescence of the human sympathetic ganglion Fig 1 Numerous strongly autofluorescent yellowish granules are also seen in the cytoplasm of the nerve cells denoted with 1 The cytoplasm of some nerve cells exhibits a weak or moderate fluorescence (arrow) Some nerve cells are without formaldehyde induced cytoplasmic fluorescence × 290

Fig 2 Two blood vessels surrounded by a lattice of greenish fluorescent adrenergic fibres projecting at various angles into the surrounding tissue are seen in the centre of the figure The cytoplasm of some nerve cells shows weak fluorescence or none (arrow) × 290

Fig 3 One larger ganglion cell is seen in the centre of the figure Closely adjacent to it a small, strongly greenish fluorescent area of adrenergic nature is seen between two white ' Fluorescent structures of this kind were seen in only 2 of the 9 ganglia studied × 5?0



Fig 5 MAO activity of the human sympathetic ganglion The cytoplasm of all the nerve cells exhibits a moderate or strong activity Incubation time 30 min ×45

which exhibited a bright greenish fluorescence identical to that seen in the nerve cells (Fig 1 and 2) Fluorescent fibres were often seen closely adjacent to the nerve cells and they were also numerous in the stroma of the ganglion tissue The delicate nerve fibres, with a distinct greenish fluorescence, were a good criterion of the management of the freeze-drying procedure

Most of the nerve cells contained numerous coarse cytoplasmic, golden yellow fluorescent granules This stable autofluorescence was in general more intense than the average catecholamine fluorescence of the cell bodies This granular fluorescent material is obviously due to lipofuscin which is characteristically present in human nervous tissue with advancing age (Samorajski *et al* 1964)

B *MAO localization*

As seen in Fig 5 almost all the ganglion cells exhibited at least moderate MAO activity in histochemical demonstration The *large ganglion cells* could be divided into two categories, the cytoplasm of some cells being moderately and that of the

Fig 4 An islet consisting of numerous small nerve cells (arrow) the cytoplasm of which exhibits traces of greenish fluorescence is seen in the centre of the figure The islet is surrounded by a thin connective tissue sheath Small islets of this kind were seen in only two ganglia. ×120

others strongly or very strongly reactive The cytoplasmic rection product was finely granular and the blue purplish precipitate was diffusely distributed in the cytoplasm of the ganglion cells In the *small ganglion cells* the average reactivity of the cytoplasm was *strong* The cytoplasm of most of the *satellite cells* gave no reaction. However, in the site of several satellite cells, small granules were seen that may have represented the mitochondrial activity of the satellite cells Most of the delicate nerve fibres of the ganglion tissue showed weak or moderate MAO activity In many thin apparently preganglionic fibres, the myelin sheath took on an intense colour In the sections preincubated in the presence of 10^{-3} M iproniazide or in the absence of the substrate, only a weakly brownish reaction was seen in the cytoplasm of the nerve cell bodies This brownish precipitate was interpreted as due to non-specific staining of the cytoplasm

C *Cholinesterase localization*

The results obtained are summarized in Table I The various substrates used were not hydrolyzed equally by cholinesterases of the ganglion cells, the satellite cells and the nerve fibres (Fig 6—11) The average reactivity of cytoplasm of the nerve

T [illegible]

Substrate + inhibitor	Cholinesterase activity		
	Nerve cells	Satellite cells	Nerve fibres
Acetylthiocholine iodide	Some strong, most moderate, some weak	Some strong most moderate some weak	Some strong others moderate or weak
+iso OMPA (10^{-5} M)	Some cells as above but *a uniform lowering of* reaction intensity	Most weak	Some moderate others *weak or none*
+284C51 (10^{-5} M)	Most none, some weak	Some moderate or none, most weak	None
+iso-OMPA+284C51	None	None	None
Propionylthiocholine iodide	Some strong most moderate some weak	Some strong others weak or moderate	Some moderate, others weak or none
+iso-OMPA	Most weak	None	None
+284C51	Most none some very weak	Some strong others moderate or none	Some weak, most none
+iso-OMPA+284C51	None	None	None
Butyrylthiocholine iodide	Some weak Most none	Some weak most none	Some weak mostly none
+iso-OMPA	Most very weak or none	None	None
+284C51	Some very weak, most none	Some weak most none	None
+iso-OMPA+284C51	None	None	None



Fig 6—11 Ch l e erases of the l n a s n pathet c gangl on Fre h 10 m ect ons
Fig 6 Total ChE a et l l chol ne × 275
Fig 7 Total ChF l r p n lthiochol ne × 230
Fig 8 AChE acetl l h och line iso-OMPA 10^{5} M × 230
Fig 9 AChE pr p n l h ochol ne + iso-OMPA 10^{5} M × 185
Fig 10 NsChE ace l h ochol ne 284C51 10^{5} M × 230
Fig 11 NsChE pr p l h ochol ne + 284C51 10^{5} M × 185

cells was moderate when acetyl or propionylthiocholine iodide was used as a substrate (Fig. 6 and 7), whereas in sections incubated in the presence of butyrylthiocholine iodide there were only a few nerve cells showing even a weak reaction. Among both *the large and the small nerve cells* there was some heterogeneity in the intensity of reaction of the cell cytoplasm. When the reaction of the nerve cell body was intense, the cytoplasm of the surrounding satellite cells mostly had a strong activity as well, and vice versa.

A combination of iso-OMPA (10^{-5} M) and 284C51 (10^{-5} M) in the incubation medium totally abolished the ChE activity of all the components of the ganglion. When 284C51 (10^{-5} M) was used alone, slight ChE activity was left in some of the nerve cells, and the cytoplasm of the satellite cells gave a positive reaction (Fig. 10 and 11). When iso-OMPA (10^{-5} M) was used, most of the activity of the satellite cells was inhibited but that of the nerve cell bodies was hardly affected (Fig. 8 and 9).

When the reaction intensity of the cytoplasm was weak (with butyrylthiocholine iodide as substrate or when inhibitors were used in various combinations) evalution of the reaction intensity of nerve cell bodies was rendered difficult by the numerous coarse lipofuscin granules in the nerve cell bodies.

2. *Quantitative Studies*

A. *MAO activity*

The mean MAO activity of the ganglia was 3.67 μmoles 4-hydroxyquinoline formed per g wet weight of tissue per hour at 37° C. The values in Table II are expressed as μmoles/mg wet weight or protein/min. The ranges were 27.4—182 $\times 10^{-6}$ and 295—1000 $\times 10^{-6}$, respectively. There were no significant differences in MAO activity in ganglia taken from patients with different diseases.

B. *Cholinesterase activity*

The ganglia showed the highest ChE activity towards ACh (Table III). The activity towards ProCh was also fairly high. BuCh on the other hand was hydrolyzed only about 26 % and 36 % of the rate of ACh and ProCh hydrolysis, respectively. Acetyl-β-methylcholine iodide (6.40 $\times 10^{-3}$ μmol/mg protein/min) and benzoylcholine iodide (<2.5 $\times 10^{-3}$ μmol/mg protein/min) were hydrolyzed even more slowly than BuCh. About 85 % of the ACh splitting activity was 284C51 sensitive, i.e. due to AChE activity. Almost as much ProCh was hydrolyzed by AChE (75 %), whereas BuCh was mainly hydrolyzed by ns ChE (71 %).

The ganglia of the arteriosclerotic group I had somewhat less ChE activity than those of the other groups. However, the former had been stored in the deep-freezer much longer than the latter and ChE was determined without preincubation (see Material and Methods), which might account for the differences. Otherwise there were no significant differences in ChE activities in ganglia taken from different sources.

TABLE II MAO activity in human sympathetic ganglion

Group	MAO Activity (×10 ⁴ μmol/mg/min) wet weight		protein	
I Arteriosclerosis 10 M & F 61±3 0 y	65 1	16 0	550	78 3
II Lupus eryth diss 1 F 23 y	77 6	—	703	—
III Arteriosclerosis 3 F 65±5 5 y	65 8	10 6	693	56 7
IV Other diseases 3 M & F 39±4 7 y	50 6	3 8	422	75 0
V Autopsy Material 4 M 52±7 7 y	52 3	5 5	581	56 7
Mean 21 M & F 54±3 3 y	61 0	7 3	566	41 7

tromb prof extr pelv

M = Male
F = Female

TABLE III Cholinesterase activity in human sympathetic ganglion

Group	Cholinesterase Activity (×10 ⁴ μmol/mg protein/min) ACh TAChE	ACh AChE	ACh ns ChE	ProCh TProChE	ProCh AChE	ProCh ns ChE	BuCh TBuChE	BuCh AChE	BuCh ns ChE
I A scl	48 9 7 1	38 5 5 8	7 6 1 9	36 9 4 7	22 9 3 6	10 3 2 0	15 1 2 4	4 9 1 1	10 6 1 7
II L e d	64 0	61 5	2 5	48 2	47 0	1 2	8 4	1 2	7 2
III A scl	81 9 4 7	74 6 4 8	8 6 1 2	—	—	—	—	—	—
IV O d	79 0 14 5	74 5 12 3	5 8 2 0	—	—	—	—	—	—
V A M	60 3 5 5	50 5 5 0	10 0 0 9	—	—	—	—	—	—
Mean	61 4 4 9	53 6 4 8	7 7 0 9	38 0 4 3	25 6 4 2	9 2 2 1	14 5 2 2	4 6 1 1	10 2 1 5

lerot c group 1 different ns Cl F) ured after = ACl

Discussion

The sympathetic ganglia are known to contain catecholamines, principally noradrenaline (Euler 1946, Vogt 1954) Since the fluorescence developed relatively rapidly, was green in colour, and disappeared in sodium borohydride treated sections, there seems little doubt that the fluorescent material in the human sympathetic ganglia is noradrenaline (Eränkö 1967)

The fluorescence in the nerve cells was much weaker than, for instance in the nerve cells of the rat superior cervical ganglion (see *e g* Härkonen 1964) or in the lumbar ganglion of the cat (Norberg and Hamberger 1964) Many of the nerve cells were totally negative, in this respect resembling the superior cervical ganglion of the cat (Hamberger *et al* 1963) Quantitative measurements of noradrenaline in the human sympathetic ganglia (1—3 μg/g tissue) and in the lumbar ganglia of the cat (12 μg/g tissue) (Euler 1956) are consistent with the low intensity of fluorescence seen in the lumbar ganglion of the man.

In its enzyme pattern the human lumbar sympathetic ganglion resembles the rat superior cervical ganglion, since all the nerve cells contained AChE and MAO activity (Härkonen 1964) The finding of some ns ChE-positive ganglion cells is at variance with that of Cauna *et al* (1961), who stated that all the nerve cells were ns ChE-negative In the rat sympathetic ganglia studied with acetylthiocholine as substrate, 55—63 % of the total ChE activity was due to AChE (Klingman *et al* 1968) This is a somewhat lower percentage than we found in human lumbar sympathetic ganglia when ACh and ProCh were used as substrates Klingman *et al* used the same inhibitor, 284C51, as we did to inhibit AChE activity specifically The total ChE activity in rat and cat sympathetic ganglia (about 27×10^{3} μmol/mg wet weight/min at 37° C) was clearly higher than the activity we observed in human ganglia ($4\,2 \times 10^{3}$ μmol/mg wet weight/min at 25° C) This is probably mainly due to the fact that acetylthiocholine is hydrolyzed by ChEs more rapidly than ACh (Koelle and Friedenwald 1949) When ChE activity was determined with ACh as substrate by a titration method very similar to ours the total ChE activity found in the superior cervical ganglion of the rat (Eranko 1970 personal communication) was 56×10^{3} μmol/mg wet weight/min and in the same organ of the cat (Holmstedt *et al* 1963) $9\,13 \times 10^{3}$ μmol/mg wet weight/min When we incubated rat and human ganglia together, the relatively higher ChE activity in the superior cervical ganglion of the rat was also evident Thus there seems to be no doubt that human lumbar sympathetic ganglia possess less ChE activity than rat and cat superior cervical ganglia At high variation in ChE activity (about 2 5 fold) in individual rat sympathetic ganglia has been reported (McLennan 1954 Klingman *et al* 1968) We found an even higher variation in human sympathetic ganglia (5 5-fold) than they found in rat ganglia. This is easier to understand since human ganglia are certainly less homogenous (greater variation in age sex and amount of connective tissue) than rat ganglia

The MAO activity in human lumbar sympathetic ganglia (3 67 μmol/g tissue/hr at 37° C) is close to the values reported by Consolo *et al* (1968) for cat ganglia

(superior cervical ganglia 6 5 μmol/g tissue/hr, lumbar ganglia 4 2 μmol/g tissue/hr at 38° C) Values about five times as high have been observed in rat superior cervical ganglia (19 7 μmol/g tissue/hr, reported by Klingman *et al* 1966).

Previous histochemical and biochemical studies indicate that the sympathetic ganglia of the cat contain two functionally distinct cell populations (Sjoqvist 1962, Giacobini *et al* 1967) In the rat, on the other hand, no sharp distinction can be drawn between adrenergic and cholinergic cells in the sympathetic ganglia (Harkönen 1964) This also seems to be the case in the human sympathetic ganglia The role of AChE in the adrenergic neurones is still unknown Burn and Rand (1962) have suggested that a cholinergic mechanism intervenes in the release of noradrenaline by postganglionic adrenergic fibres They stated that impulses in the adrenergic nerve first liberate ACh at the terminals and that this in turn liberates noradrenaline If the activity of ACh in the nerve cells corresponds to the extent of ACh participation in transmission, the AChE activity observed in human adrenergic ganglion cells could be explained by Burn and Rand's theory Alternative hypotheses have been presented to account for the existence of AChE in adrenergic nerve cells, Nachmansohn's theory (1959) of the universal role of ACh in conduction the phylogenetic theory of neurones which lose their cholinacetylase but not their AChE activity (Koelle 1955 Feldberg 1957) and the possibility that some other substrate exists for AChE besides ACh (Feldberg 1957)

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From the Department of Zoophysiology, University of Goteborg, Sweden, and Department of Physiology, University of Iceland, Reykjavik

The Effects of K⁺-free Solution on Tension Development in the Smooth Muscle Taenia Coli from the Guinea Pig

By

JÓHANN AXELSSON and BO HOLMBERG

Received 10 December 1970

Abstract

AXELSSON, J and B HOLMBERG *The effects of K^+-free solution on tension development in the smooth muscle Taenia Coli from the guinea pig* Acta physiol scand 1971 *82* 322—332

In the smooth muscle taenia coli from guinea pig changes in the pattern of electrical and [illegible] ons The ability to develop [illegible] K^+ and Na^+ The changes [illegible] nce with metabolic energy [illegible] ssive mechanial properties An interference with the normal functions of Ca^{2+} in the excitation contraction coupling is suggested

In the smooth muscle of the guinea-pig taenia coli exposure to K^+-deficient solutions changes the pattern of electrical and mechanical activity and the relationship between the two (Holman 1958, Axelsson 1961, Bulbring and Kuriyama 1963)

The present work aims at analysing these effects Some of the results have been communicated to the XIII Scandinavian Congress of Physiology (Axelsson *et al*, 1969)

Methods

Dissection and pretreatments

Guinea pigs were stunned with a blow and bled out The cecum was immersed in normal Krebs solution (see below) at 37° C with adrenaline 2×10^{-6} g/ml to abolish spontaneous activity Pieces of taenia of about 25 mm were dissected and transferred to a dish containing normal Krebs solution Small stainless steel hooks were tied to each end of the muscle used for iso- [illegible] containing 50 ml normal Krebs solu [illegible] f the tissue was attached to a plastic [illegible] via a plastic pin The length of the [illegible] h end of the muscles used for deter [illegible] mounted in a plastic muscle holder [illegible] and Axelsson 1965) Whereafter the [illegible] s solution *Lo* was again determined 15 min before beginning the experiments All experiments were done at 37° C

Solutions

The compositions of the various solutions are described in the table Concentrations in mmole/l (mM).

Solution	K^+	Na^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-	$H_2PO_4^-$	Glucose
Normal Krebs	5 93	137 47	2 49	1 19	134 11	15 48	1 19	11 5
High K⁺	127.94	15 48	2 49	1 19	134 11	15 48	1 19	11 5
K⁺-free	0	143 40	2 49	1 19	134 11	15 48	1 19	11 5
High K⁺ (Na⁺-free)	143 40	0	2 49	1 19	134 11	15 48	1 19	11 5

The solutions were prepared with double distilled water and chemicals of Pro Analysi quality They were bubbled with a mixture of O_2 (97 %) and CO_2 (3 %) and pH was about 7 4

Recording technique

The force displacement transducer used for isometric recording of muscle tension was Grass FT 03 The output potential was recorded on Beckman/Offner Dynograph R B or Grass Polygraph

For simultaneous recording of electrical and isometric mechanical activtiy the sucrose gap method was used (Stampfli 1954).

Stimulation

[illegible] cle [illegible] mal [illegible]
supramaximal stimulus

High external K⁺ The relation between the membrane potential of taenia coli and the external K⁺ concentration has been investigated by many authors Casteels and Kuriyama (1966) found that the membrane potential decreased from −57 to −9 mV when the external K⁺ concentration was changed from 5 9 to 118 mM Maximum mechanical response was obtained already with 118 mM K⁺ The concentration used as stimulus in the present experiments was 128 mM K⁺

Determination of ion content

About 10 muscle pieces from each animal were divided in groups and each muscle was analysed after incubation in normal or K⁺-free solution Before analysis the muscles were wiped on a Perspex sheet and their wet weight determined

Na⁺ and K⁺ The m[illegible]
subjected to stimulation [illegible]
120 and 150 min For d[illegible]
more than 1 hr about 1[illegible]
weighed (wet weight) and placed in a pot of quartz 1 ml HNO_3 (sp gr 1 40) and 1 ml
[illegible]
0 88) at 70° C Chloride in this solution was determined with an Aminco [illegible] chloride titrator

Ca^{2+} and Mg^{2+} The muscles were mounted in isotonic conditions with a constant [illegible]
[illegible]
(TCA) and a [illegible]
tubes were tre[illegible]
and double dis[illegible]
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tometer

Determination of the water content

The fresh weight of the tissue was determined immediately after dissection and the wet weight after exposure to normal Krebs or K free solution Before weighing the muscle was [illegible] a Perspex sheet The dry weight was obtained by drying the tissue for 15 hrs at [illegible]

Analysis of adenosine triphosphate (ATP) and creatine phosphate (CP)

From each animal 8 muscles of about 25 mm were free dissected weighed and divided in two groups Each muscle was mounted in a plastic holder at L_0 After incubation in normal and K^+ free solution respectively the tissue was transferred into Frigen ($CFCl_3$) cooled to −60° C After 15 sec the frozen tissue was cut free, freeze dried at −30° C for 3 hrs and stored at −20° C up to 5 days Each sample was homogenized in 0 8 ml 6 % icecold perchloric acid (PCA) and centrifuged 10 min at 5000 g The supernatant was neutralized with 5 M K_2CO_3 and used for enzymic spectrophotometric analysis of adenosine triphosphate (ATP) and creatine phosphate (CP) (Beviz unpublished) The content of ATP and CP of the freezedried muscles were not decreased during the storage

Glycerinated taenia

The preparation of glycerinated pieces was described by Axelsson, Holmberg and Hogberg (1965) *Before glycerol extraction the muscles were exposed for 3 hrs to K^+ free and normal* Krebs solution respectively

Controlled stretching

The method is described in detail by Högberg (1969) Before each experiment the glycerinated muscles were mounted in an organ bath at 37° C under 30 dynes tension and allowed to recover for 30 min in high K^+ (Na^+ free) and K free solution respectively

Intact muscles were mounted in similar way but before stretching they were exposed to Ca^{2+} free solutions containing EGTA (1 mM) for 30 min

The rate of stretch was in all experiments 0 2 mm/sec The stress at various lengths was for each muscle calculated as described by Aberg and Axelsson (1965)

All results presented here are expressed as the mean value ± S D if not otherwise stated

Results

The effects of K free solutions on spontaneous activity

Mechanical activity was studied in isometric condition at L_0 In normal solution it consisted in contractions occurring at varying but for each muscle constant intervals The peak tension reached in these contractions was similar to the maximum tension developed *in response to depolarization in high K solution*

Exposure to K free solution changed this *pattern into* continuous activity resembling complete or incomplete tetanus This pattern was maintained for ca 15 min, during which the tension development declined rapidly and ceased after 15 min exposure to K free solution For the following 50 ± 10 min no activity was *recorded* Then *in all experiments mechanical activity reappeared* and lasted for further *60—90 min It consisted in small shortlasting contractions at constant inter*vals The tension development reached its peak value (about 25 % of that *in normal* solution) after about 2 hrs exposure to K free solution whereafter it declined and ceased After 3 hrs exposure to K free solution the muscles were returned to normal solution. Spontaneous mechanical activity then reappeared after ca 30 min Sucrose gap recordings showed *corresponding changes in the pattern* of the electrical activity After change to K -free solution there was an *initial but shortlasting depolarization* with increased frequency of spike discharge Consequently the phasic tension responses to spikes fused but no contracture developed The membrane potential then slowly increased and the frequency of spike discharge decreased and ceased after about 15 min After *approximately* 1 hr s exposure spontaneous electrical activity of reduced amplitude reappeared and *continued for about* 1 hr

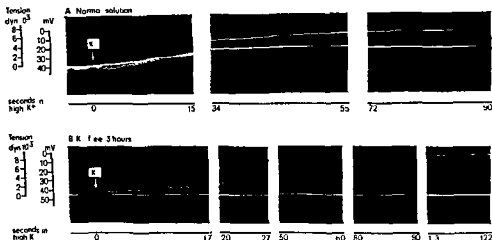


Fig 1 The effect of high K on the electrical and mechanical activity of taenia coli A Normal Krebs solution B After 3 hrs in K free solution Upper record Electrical activity Lower record Tension

These experiments confirmed and extended earlier observations on changes in the pattern of electrical and mechanical activity but were inconclusive as to whether, or to what extent their relationship is changed in K free solution

Responses to stimulation

When carbachol (10^{-6} g/ml) was used as stimuli (see Methods) the muscles were exposed to the drug for 2 min each time at intervals of 30 min In K free solution the tension response declined rapidly After 1 hr s exposure it was only 20 % of that in normal solution and after 2 hrs it was 5 % and stable After 30 min in normal solution and previous exposure to K free solution for 3 hrs the tension response had recovered by about 70 % Sucrose gap recordings revealed that the depolarizing effect of carbachol fell during exposure to K free solutions The similar timecourse of the two processes suggests that the fall in the mechanical response in K free solution was secondary to the reduction of the depolarizing effect of the drug Similar observations were made with acetylcholine by Bulbring and Kuriyama (1963)

When depolarization by high K solution (see Methods) was used as stimulus the muscles were stimulated for 5 min After each exposure to high K the muscles were allowed to recover for 1 hr in normal solution before an experimental variable was changed The depolarization induced by high K solution was not significantly changed during exposure to K free solution (see Fig 1) while the mechanical response was greatly reduced Fig 2 shows the maximum tension developed in response to high K solution after exposure to K free solution for different length of time and during recovery in normal solution The tension is expressed as percentage of that in normal solution After 3 hrs exposure it was reduced to 13 ± 8 % After 1 hr in normal Krebs solution the tension response had fully recovered

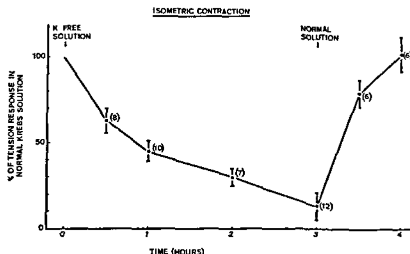


Fig 2 The maximum tension response to high K^+ in K^+ free and normal Krebs solution Ordinate Force expressed as percentage of the maximum tension response to high K^+ in normal Krebs solution Abscissa Time in hours Means ± S D Number of muscles in brackets

In a series of experiments the [Ca^{2+}] in the high K^+ solution was raised from the normal 2 5 mM to 5 respectively 20 mM Otherwise the experiments were performed as described above The results are summarized in Table I

Thus doubling the [Ca^{2+}] in the depolarizing solution counteracted to some extent the effects of K^+-free solution on the tension response Further increase in the [Ca^{2+}] caused no further improvement

In another series of experiments, summarized in Fig 3, the tension was recorded during 1 hr's exposure to high K^+ The first group (A) had been in normal solution and the tension response decreased to 58 ± 9 % The second group (B) had been exposed to K^+ free solution for 3 hrs and the tension response increased from 13 ± 8 to 35 ± 18 % The tension response was expressed in per cent of the maximal tension response to high K after incubation in normal Krebs solution

The mechanical responses to electrical stimulation during exposure to K^+-free solution followed a pattern similar to that described above for stimulation by carbachol

TABLE I Maximum tension response to high K^+ solution containing varying concentrations of Ca^{++} Tension expressed in per cent of that in normal Krebs solution Means ± S D N = number of experiments

Ca^{++} conc in the high K^+ sol	Krebs	K^+ free 3 hours	N
2 5	100	13 ± 8	12
5 0 mM	108 ± 10	47 ± 11	11
20 0 mM	114 ± 14	41 ± 14	11

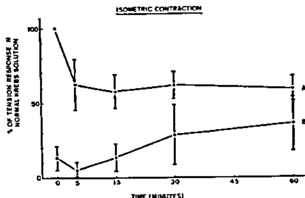


Fig 3 Tension development in high K^+ solution after previous incubation in A normal Krebs solution B 3 hrs in K^+ free solution Ordinate Force expressed in per cent of the maximum tension response to high K^+ in normal Krebs solution Abscissa Time in high K^+ Means ± S.D of 12 muscles

The effect of K^+-free solution on ion and water content

The changes in intracellular ion composition during exposure to K^+-free solution was studied in an attempt to establish a correlation between the ion content and the decreased ability to develop tension

Na and K^+ The K^+ and Na^+ content was determined as described in Methods after 15, 30 60, 90, 120 and 180 min in K^+ free solution The results are shown in Fig 4 During 3 hrs incubation in K^+ free solution the K^+ content decreased from 74 ± 8 to 3 ± 1 mmoles/kg wet weight and the Na^+ content increased from 80 ± 7 to 156 ± 11 mmoles/kg wet weight After previous incubation for 3 hrs in K^+ free solution the K^+ and Na^+ content of several muscles was determined after 15, 30 and 60 min in normal Krebs solution The K^+ content increased to 71 ± 11 mmoles/kg wet weight and the Na^+ content decreased to 78 ± 13 mmoles/kg wet weight Thus the changes in K^+ and Na^+ content after exposure to K^+-free solution were fully reversible and the sum of K^+ and Na content approximately the same throughout the experiment

In a series of experiments the K^+ and Na^+ content of the tissue was determined in conditions identical to those described in Fig 3 After 2 5, 15 30 and 60 in high K^+ solution the ion content was analysed Before analysis the muscles were dipped into 9 % sucrose solution of 37° C The results are summarized in Fig 5 and 6

When the muscles had been preincubated in normal Krebs solution the changes in K^+ and Na content were small after the first 2 min in high K^+ solution (Fig 5) On the other hand when the muscles had been previously exposed to K free solution for 3 hrs the K content was still increasing and the Na content decreasing after 15 min in high K solution (Fig 6)

Cl content In two series of experiments with 10 muscles in each series the content of Cl was determined after 3 hrs incubation in normal Krebs solution and K^+-free solution respectively The Cl content was 93 3 ± 4 9 mmoles/kg wet weight and 90 4 ± 5 4 respectively

Ca^{++} and Mg^{2} content The muscles were mounted in isotonic conditions and Ca^{++} and Mg^{++} content was determined after 3 hrs incubation in K^+-free solution and

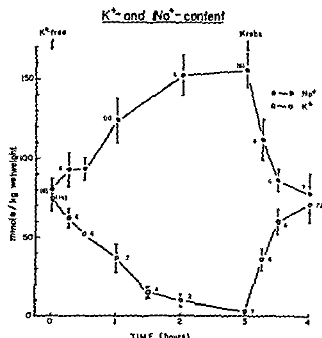


Fig 4 The K and Na content of taenia coli in K⁺ free and normal Krebs solution Means ± S D Number of determinations in brackets

normal Krebs solution respectively The results are summarized in Table II As can be seen from the table there was no significant difference in Ca⁺ and Mg⁺ content after 3 hrs exposure to K free solution compared to normal Krebs

The changes of the water content of the tissue were studied in isometric conditions by comparing the dry weight/wet weight and wet weight/fresh weight ratios after 3 hrs incubation in normal Krebs and K free solution respectively The results are summarized in Table III No difference was found in the ratios dry weight/wet weight and wet weight/fresh weight after 3 hrs exposure to the respective solutions

Passive mechanical properties

Changes in intracellular K or Na might affect passive mechanical properties of muscles Barr *et al* (1962) reported that increased [K] had a plasticizing effect on the contractile apparatus Studies of length tension relationship and stress relaxation of inactive muscles revealed no significant difference between muscles incubated for 3 hrs in normal Krebs and K free solution respectively The same held true for glycerinated muscle pieces

The effect of K free solution on the content of energy rich phosphate compounds

(In cooperation with A Beviz)

As K⁺ is known to activate many transphosphorylating enzyme systems (for references see Ussing 1960) the reduced tension response to stimuli after exposure to K free solution might conceivably be related to changes in the metabolic processes

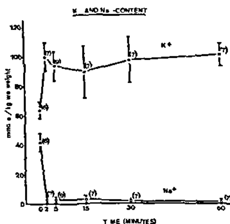


Fig 5

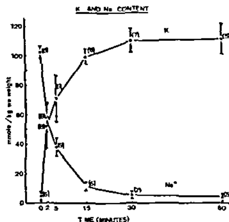


Fig 6

Fig 5 The K^+ and Na^+ content of taenia coli during 1 hr's exposure to high K^+ after pre incubation in normal Krebs solution The muscles were dipped into 9 % sucrose solution before analysis Means ± S D Number of experiments in brackets

Fig 6 The K^+ and Na^+ content of taenia coli during 1 hr's exposure to high K^+ after 3 hrs preincubation in K^+ free solution The muscles were dipped into 9 % sucrose solution before analysis Means ± S.D Number of experiments in brackets

TABLE II The content of Ca^{++} and Mg^{++} in taenia coli after exposure to normal Krebs and K^+ free solution Concentrations in mmoles/kg wet weight Means ± S D Number of experiments 10

	Krebs 1 hour	Krebs 4 hours	Krebs 1 hour K^+ free 3 hours
Ca^{++}	3 22 ± 0 40	3 65 ± 0 51	3 38 ± 0 35
Mg^{++}	4 84 ± 0 26	5 12 ± 0 31	5 44 ± 0 47

TABLE III The ratios dry weight/wet weight and wet weight/fresh weight of taenia coli after 3 hrs exposure to normal Krebs or K^+ free solution Means ± S D N = number of experiments

Medium	dry weight wet weight	N	wet weight/fresh weight	N
Krebs 3 hrs	0 165 ± 0 009	12	1 079 ± 0 065	16
K^+ free 3 hrs	0 169 ± 0 008	13	1 078 ± 0 032	16

which provide energy for contraction In a series of experiments the concentrations of adenosine triphosphate (ATP) and creatine phosphate (CP) were determined after 3 hrs in normal Krebs and K free solution respectively No significant changes in the concentrations of ATP and CP were detected It was not excluded however that the rate of processes providing energy for muscular contraction was reduced after exposure to K free solution Therefore in another series of experiments the concentration of ATP and CP was determined when maximum tension had devel

TABLE IV The concentration of ATP and CP after 3 hrs exposure to normal Krebs and K^+ free solution respectively and one minutes depolarization in high K^+ solution. Concentration in µmoles/g fresh weight. Means ± S.D. N = 12. P = probability that the difference was due to chance

Medium	ATP	CP
Krebs	1.48 ± 0.13	0.97 ± 0.29
K^+ free	1.35 ± 0.17	0.76 ± 0.29
	$P < 0.05$	$P < 0.05$

after 1 min stimulation by high K^+ solution. Previous to stimulation the muscles had been exposed for 3 hrs to normal Krebs and K^+ free solution respectively. The results are summarized in Table IV.

The effect of K^+ free solution on maximum tension response of glycerol treated taenia coli

In an attempt to assay a possible effect of K^+ independent of membrane activity on the contractile mechanism pieces of glycerol treated taenia coli were used. ATP was added to a concentration of 2 mM and the tension development was recorded. The results are summarized in Table V.

No significant difference between the maximum tension response to added ATP in K^+ free and high K^+ (Na^+ free) solution was found.

Discussion

The changes in the pattern of spontaneous electrical and thereby mechanical activity in K^+ free solutions may to some extent be accounted for by changes in K^+ conductance. In various tissues exposure to K^+ free solution has been found to decrease K^+ permeability (Keynes and Lewis 1951, Carmeliet 1961, Wahlström 1969). The initial depolarization and increase in the frequency of spike discharge observed in the present experiments is consistent with such change. In taenia coli however the excitation was shortlasting and followed by an increase in membrane potential and cessation of spike discharge. In the same tissue Casteels (1970) found after 20 min exposure to K^+ free solution a progressive increase in K^+ efflux which explains the hyperpolarizing phase. The second outburst of spontaneous activity is still to be accounted for.

TABLE V Maximum tension response (dyn/mm²) of glycerinated taenia coli to added ATP (2 mM) in high K^+ (Na^+ free) or K^+ free solution. Means ± S.D. Number of experiments 23

Solution	High K^+ (Na^+ free)	K^+ free
Tension dyn/mm²	250 105	220 114

Although the present, as well as previous results strongly suggested a change in the relationship between electrical and mechanical activity, is was not excluded that in the experimental conditions referred to, changes in conductance, syncronization or other electrical properties might to some extent account for the apparent dissociation The experiments in which supramaximal stimuli of various kinds were applied showed, however, that the ability to develop tension fell parallel with a fall in the intracellular K^+ content and a corresponding rise in the content of intracellular Na^+ The water content of the cells did not change during exposure to K^+-free solution nor did extracellular space (Holmberg and Wahlström unpublished) Thus the estimations of K^+ and Na^+ content reflect the intracellular concentrations of these ions In normal solution the recovery of the tension response and the K^+ and Na^+ content developed parallel Axelsson *et al* (1965) studied quantitatively the effects of reduced supply of metabolic energy on the tension responses to supramaximal stimuli. Their results bear striking similarity to those obtained in K^+-free solution But after 1 hr in glucose-free solutions the tissue content of ATP and CP is reduced by at least 50 % (Bueding *et al* 1967) while in the present experiments no significant change was detected after 3 hrs in K^+ free solution. During stimulation there was however a small decrease in the ATP and CP content of tissues preincubated for 3 hrs in K free solution We are satisfied that neither changes in electrical activity, electrical responses to stimulation, nor reduction in metabolic energy can account for the reduced development in K^+ free medium

Briggs (1963) reported that the tension development of glycerinated guinea pig uterine fibres was reduced by 23 % in solutions containing Na^+ instead of K^+ These effects however, might as well be due to an inhibiting effect of excess Na^+ as to lack of K In the present experiments no significant difference was found in the maximum tension response of glycerinated muscles to added ATP in the two solutions Possible effects might however be masked by the great reduction of tension development of a glycerol treated muscle compared to a living muscle

In the intact taenia Ca^2 is supposed to play a role in the activation of the contractile mechanism similar to that established for striated muscle Without going further into the complex problem of ion competition at binding sites, a Ca^2 —Na^+ antagonism has been suggested in the heart (Luttgau and Niedergerke 1958) and in taenia coli (Goodford 1966) In the present experiments raising the $[Ca^{+}]$ in the depolarizing solution counteracted to some extent the effects of K free solution on tension It is suggested that reduced intracellular K or Na excess may interfer with the normal functions of Ca^+ in the excitation contraction coupling

The determinations of Ca^2 and Mg^2 were made by one of us (B Holmberg) at Dr P J Goodford's laboratory at the Wellcome Foundation Beckenham England His generous help is gratefully acknowledged

We are grateful to Mrs Gunilla Rydgren for excellent technical assistance

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From the Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden

Efflux of Prostaglandin E_2 from Cat Paws Perfused with Compound 48/80

By

ERIK ÄNGGÅRD and KJELL STRANDBERG

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Abstract

ÄNGGÅRD, E and K STRANDBERG *Efflux of prostaglandin E_2 from cat paws perfused with compound 48/80* Acta physiol scand 1971 *82* 333—344

hydroxy prostaglandin dehydrogenase from swine lung in the presence of NAD+ complete

material appearing in connection with histamine release in the cat paw induced by compound 48/80

Histamine and lipid soluble smooth muscle stimulating principles appear in the effluent when cat paws are perfused with compound 48/80 (Chakravarty Hogberg and Uvnas 1959, Anggård *et al* 1963) The lipid soluble spasmogenic material has been designated 'slow reacting substance (SRS) since it produces slow, sustained contractions of the isolated guinea pig ileum Fractionation of this material on silicic acid yielded two major components with different biological properties (Anggård *et al* 1963) The SRS response (*vide supra*) is produced by the more polar component This has recently been further purified and it seems to be an unsaturated hydroxy carboxylic acid (Strandberg and Uvnas 1971)

In the present paper the nature of the less polar smooth muscle stimulating com-

ponent was studied Comparative biological assay, enzymatic analysis with 15-hydroxy prostaglandin dehydrogenase as well as various chromatographic procedures showed that the compound was identical with prostaglandin E_2

Methods and Materials

Cat paw perfusion Paws were taken from cats (1 5—5 0 kg) killed by an injection of pentobarbital intraperitoneally They were prepared and mounted in temperature controlled perfusion chambers as described elsewhere (Strandberg 1971) In some experiments, one paw of each pair (fore or hind) served as control and was perfused with a salt solution alone during the entire perfusion period (100 min) The other paw(s) was perfused with the same salt solution for an equilibration period (20 min) Then compound 48/80 (1 μg/ml) was added to the perfusion medium and the perfusion was continued for 80 min The infusion rate was 1 ml/min and the temperature was 27° C The composition of the perfusion medium was NaCl, 154 mM, KCl, 2 7 mM, $CaCl_2$ 0 9 mM containing 10 per cent (v/v) Sorensen phosphate buffer (Na_2HPO_4+KH_2PO_4, 67 mM) pH 7 0 The effluents were collected in 20-min fractions in ice-chilled glass tubes The fractions collected after the introduction of compound 48/80 were combined as were the corresponding fractions from the control paw The fractions were centrifuged to remove any blood cells present, briefly boiled, filtered and lyophilized

Extraction and solvent partition Each lyophilized perfusate was extracted twice with 80 per cent aqueous ethanol, 50 ml and 25 ml respectively After filtration the volume of the extract was reduced to about one fifth by evaporation under reduced pressure at room temperature This solution was adjusted to pH 3 by the addition of 1 N HCl and three times extracted with two volumes of ether The ether extract (acidic lipid extract) was either applied directly to a silicic acid column prepared for the methanol chloroform system (*vide infra*) or washed with water until neutral and evaporated to dryness *in vacuo* The recovery of non histamine spasmogenic activity in this extraction procedure is about 70 per cent (Strandberg and Uvnäs 1971) When used as tracer a known small amount (2—10 ng) of ^{3}H-labelled $PGF_{2\alpha}$ (Änggård Green and Samuelsson 1965) with a specific activity of 4 5 C/mmole was added to the alcoholic extract In these experiments the aqueous phase was extracted twice with two volumes of ether which were discarded before acidification The radioactivity was determined by conventional liquid scintillation counting using a Packard Tri Carb liquid scintillation spectrometer

Chromatographic procedures

1 *Silicic acid chromatography* (a) Methanol chloroform (M C) system Chromatography was performed on 2 g columns of silicic acid (Unisil, 100—200 mesh, Clarkson Chemical Company) activated at 115° essentially as described previously (Änggård *et al* 1963) The acidic lipid extract (the combined ether phases) was applied on the column and a discontinuous gradient elution system of increasing concentrations of methanol in chloroform was used at 4° C Fractions of 10 ml were collected (b) Ethyl acetate benzene (E B) system Columns containing 1 g of silicic acid (Mallinckrodt 100 mesh) activated at 115° C were prepared The acidic lipid extract was applied on the column in ethyl acetate benzene (1 9) Prostaglandins were eluted from the column either by stepwise increasing the concentration of ethyl acetate in benzene (Samuelsson 1963) or by a linear gradient of ethyl acetate in benzene starting with 1 9 and finishing with a 9 1 volumetric ratio (Bygdeman and Samuelsson 1966) In both instances remaining lipid material was eluted by 25 ml of methanol Fractions of 10 ml and 1—2 ml respectively were collected

2 *Reversed phase partition chromatography* This was performed on 4 5 g of hydrophobic Supercel using 4 ml of isooctanol chloroform (1 1) as the stationary phase and 300 ml of methanol water (1 1) as the moving phase (C-50) (Norman 1953, Bergström *et al* 1963) The analogous system C-47 was also used in some experiments Fractions of 2 0 3 5 ml were collected

3 *Thin layer chromatography (TLC)* TLC was performed principally as described by Green and Samuelsson (1964) Chromatographic plates were prepared by spreading a mixture of 30 g of Silica gel H (E Merck and 60 ml of water with or without 1 g of silver nitrate on glass plates of dimensions 50 × 200 mm or 200 × 200 mm The thickness of coating was approximately 0 25 mm The plates were activated at 115° C for one hour before use The solvent systems used are given in Table 1 Samples and reference PGE_1 (10—20 μg) and $PGF_{2\alpha}$ (6—20 μg) were applied to the plates as single spots or as bands by means of micropipettes The reference

TABLE I Solvent systems used in thin layer chromatography

System	Composition	Adsorbent
A II	Ethyl acetate acetic acid methanol 2,2,4 trimethylpentane water (110 30 35 10 100)*	Silica gel H $AgNO_3$ (30 1)
A III	Ethyl acetate acetic acid 2,2 4 trimethylpentane water (100 10 30 100)*	Silica gel H $AgNO_3$ (30 1)
A V	Benzene dioxane acetic acid (65 15 2)	Silica gel H

* The solvent mixture was equilibrated for 2 hrs and the upper phase was used

compounds were both applied to preparative plates and to separate marker plates The plates were developed until the solvent had reached between 12 and 14 cm from the origin After developing the plates the marker plate was sprayed with 10 per cent ethanolic phosphomolybdenic acid and heated at 115° C for 15 min.

The preparative plate was divided into zones corresponding to the prostaglandins on the marker plate The preparative plate could later be sprayed with the reagent to check the posi

terms of the free base

Biological assays The contractions of the smooth muscles were recorded isotonically on a smoked drum using a frontal writing lever Washing

reached, usually after 30—90 sec Total cycle time

used for uterine horns and a 4 ml one for the other

solvent was assayed after evaporation of the solven

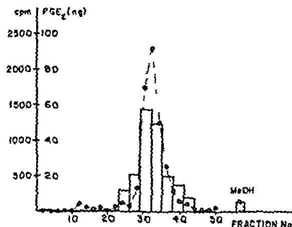


Fig 4 Silicic acid chromatography of spasmogenic lipid material from cat paw perfusates (4 cats) Silicic acid 1 g Linear gradient of ethyl acetate benzene in volumetric ratios from 1 9 to 9 1 Fractions 1 2 ml Biological activity (rat uterus) is denoted by columns Radioactivity due to added ^3H PGE_2 (○—○) was measured in aliquots of the fractions

from the ethyl acetate benzene (6 4) fractions indicating that the active principle(s) could be a PGE-compound

The combined ethyl acetate benzene (6 4) fractions were subjected to thin layer chromatography on $AgNO_3$ impregnated silica gel (Green and Samuelsson 1964) PGE_1 and PGE_2 were used as references As shown in Fig 3 most of the biological activity, as assayed on both guinea pig colon and rat uterus, was recovered from the zone corresponding to PGE_2

Further evidence as to the identity of the smooth muscle stimulating lipid was obtained by chromatographing the original acidic lipid extract together with ^3H PGE_2 on silicic acid using the linear gradient E B system (5 expts) One such chromatography is shown in Fig 4 It is seen that the biological activity appears concomitantly with the radioactivity due to the internal standard of ^3H PGE_2 Reversed phase partition chromatography (system C 50) of this material showed

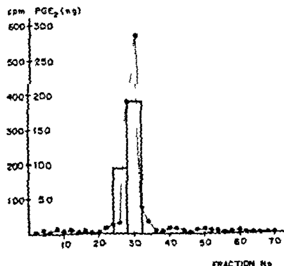


Fig 5 Reversed phase partition chromatography of spasmogenic material isolated from perfusates by extraction and silicic acid chromatography (8 cats System C-50 Fractions 2 4 ml Biological activity is denoted by columns Radioactivity due to added ^3H PGE_2 (○—○)

TABLE II Inactivation of smooth muscle stimulating material with 15 hydroxy prostaglandin dehydrogenase (PGDH)

Additions			Assay
perfusate extract	PGDH (0 2 mU)	NAD^+ (5 mM)	(ng of PGE_2)
+	+	+	0
+	—	+	17
+	+	—	17
+	—	—	20
—	+	+	0

that the spasmogenic factor appeared in a single peak which coincided with that due to ^3H PGE_2 (Fig 5) With this procedure separation of the individual prosta glandin E compounds is achieved (Bergstrom *et al* 1962)

Enzymatic identification 15 Hydroxy prostaglandin dehydrogenase isolated from swine lung is specific for the 15 (S) hydroxy group in the prostaglandins (Änggård and Samuelsson 1966 Nakano, Änggård and Samuelsson 1969) The resulting 15 keto metabolites possess low biological activity compared to that of the parent compounds (Änggård 1966, Pike, Kupiecki and Weeks 1967) When the prosta-glandin like material isolated from the perfusates was incubated with prostaglandin dehydrogenase and NAD^+ a complete biological inactivation was observed (Table II) Controls retained full activity when either the enzyme or NAD was omitted This finding constitutes further strong evidence as to the prostaglandin nature of the compound under investigation

Efflux of PGE_2 in relation to histamine and SRS In three experiments the output of histamine, SRS and PGE_2 from paws perfused with or without compound 48/80 were compared (Table III) No quantitative relationship between the appearance of these compounds was observed Small amounts of histamine and SRS but no PGE_2 were detected when the paws were perfused with salt solution alone

TABLE III Histamine, SRS and PGE_2 in the effluents from cat paws perfused with or without compound 48 80 PGE_2 was assayed on the rat uterus after preceding purification (silicic acid chromatography) No corrections for losses of PGE_2 during the purification procedures have been made

No	Experiment	Number of pooled effluents	Histamine (µg)	SRS (units)	PGE_2 (ng)
1	Control	4	6 0	5 000	NM
	Compound 48 80	4	46 3	80 000	16
2	Control	2	4 3	NM	NM
	Compound 48 80	2	56 9	191 300	7
3	Control	4	8 7	4 420	NM
	Compound 48 80	4	71 4	7 400	34

NM = not measurable

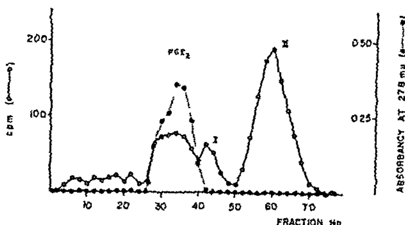


Fig 6 Reversed phase partition chromatography of ...

Metabolism of ^{3}H-PGE$_2$ in cat paw The amounts of PGE$_2$ in the effluents were generally low ($\leqslant$ 10 ng/paw) This could be due either to a low degree of formation or to a metabolic degradation of PGE$_2$ To test the latter hypothesis ^{3}H-PGE$_2$ was added to the perfusion medium as described under Methods The recovery of radioactivity in the effluent was 59—67 per cent ($n = 4$) The acidic lipids in the effluent and in the paws were extracted with ethanol and ether and subjected to reversed phase chromatography (system C 47) The result of one experiment is shown in Fig 6 The radioactivity appeared in three peaks One coincided with the reference PGE$_2$ and thus represents unmetabolized material The other two appeared with elution volumes characteristic of 11α, 15 dihydroxy-9 ketoprost-5-enoic acid (dihydro-PGE$_2$) and 11α hydroxy-9,15-diketoprost-5-enoic acid (15-keto-dihydro-PGE$_2$) respectively Furthermore, the radioactive peaks cochromatographed with the authentic metabolites on thin layer chromatography (system A III and A V)

Some variations in the peak heights were seen in the different experiments However, in all experiments considerably more 15-keto-dihydro-PGE$_2$ appeared than dihydro-PGE$_2$ The same pattern was observed whether the extracts chromatographed were prepared from the effluents or from the paws Administration of compound 48/80 had no influence on the amount of ^{3}H PGE$_2$ metabolized

Determination of esterified arachidonate in cat skin Since arachidonic acid is the biosynthetic precursor of prostaglandin E$_2$ the occurrence of this and related unsaturated fatty acids in the cat skin ($n = 4$) before and after treatment with compound 48/80 was investigated The pattern of fatty acids esterified to phospholipids in the cat paw is shown in Fig 7 It is seen that the major unsaturated C$_{20}$ acid is arachidonic acid, with small amounts of bis homo-γ-linolenate and eicosapentaenoate also being present No differences in the ratios between arachidonate and lipid phosphorus before and after compound 48/80 were observed

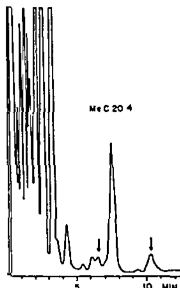


Fig 7 Gas liquid chromatography of fatty acids esterified to phospholipids in cat skin after preceding extraction and methanolysis Cond tions 10 % EGSS-X, temperature

Discussion

It was previously known (Änggård *et al* 1963) that the lipid soluble smooth muscle stimulating material appearing in the effluent from cat paws perfused with the histamine releasing agent compound 48/80 consisted of two components with different biological and chemical properties One component stimulated several smooth muscle organs and was eluted by small amounts of methanol in chloroform on silicic acid chromatography The other component designated SRS required 20—40 per cent methanol in chloroform for elution and on purification gave a slow contraction of the guinea pig ileum. The present study aimed specifically at the characterization of the former compound It was shown using parallel bioassay a variety of selective chromatographic procedures and enzymatic analysis that this compound is identical with prostaglandin E_2

Formation and release of prostaglandin like material has previously been shown to be induced by histamine liberators such as antigen (Anggard *et al* 1963 Piper and Vane 1969) venoms (Vogt *et al* 1969 Ladinsky and Strandberg 1969) d tubocurarine (Laity 1969) morphine and pethidine (Laity and Moore 1970) However, in contrast to these agents compound 48/80 in the concentration used (1 μg/ml) has not been reported to act on any other cell than the mast cell Therefore the efflux of PGE_2 from the cat paw might either originate from the tissue mast cells or be secondary to the release of histamine SRS or some unknown factor In support for the latter explanation is the finding that prostaglandin like material has been shown to appear in the effluent from guinea pig lungs perfused with partially purified SRS (guinea pig lung) histamine produced no such effect (Piper and Vane 1969)

The rate of prostaglandin formation is probably dependent on the rate of hydrolysis of precursor acids (Lands and Samuelsson 1968, Vonkeman and Van Dorp 1968) These are present mostly esterified to the 2 position in phospholipids In agreement with this view is the finding that venoms containing phospholipase A stimulates prostaglandin formation in a variety of tissues (Eliasson 1959, Vogt *et al* 1969) It is of interest here to recall the suggestion by Chakravarty *et al* (1959) that the release of histamine and formation of SRS in the cat paw by compound 48/80 is accompanied by an activation of an endogenous phospholipase A Our results reporting in the same system the occurrence of PGE_2, which is known to originate from phospholipid bound arachidonate (Lands and Samuelsson 1968 Vonkeman and Van Dorp 1968), is in agreement with this view As expected, the major unsaturated C_{20} acid present in the cat paw was arachidonate, the precursor of PGE_2 The amounts present were in the range of 18—41 μg/g tissue, thus far in excess of the amount of prostaglandin formed in the paws It is therefore likely that only a small pool of this arachidonate is actually available for prostaglandin formation by the microsomal prostaglandin synthetase Also no significant difference was observed in the ratio between arachidonate and lipid phosphorus before and after exposure to compound 48/80

Prostaglandins undergo metabolism in the cat paw as shown by the fate of infused ^3H PGE_2 The major metabolite was found to be 15-keto-13,14-dihydro PGE_2 The 15-keto metabolites of prostaglandins have low biological activity compared to that of the parent compounds (Änggård 1966 Pike, Kupiecki and Weeks 1967 Kloeze 1969 Metabolic degradation of prostaglandins in the tissues may therefore be an explanation for the relatively low amounts of prostaglandins detected in the effluents

Prostaglandins and SRS are lipid soluble acidic compounds Since SRS is more polar than PGE_2 it was interesting to note that no polar radioactive metabolites were detected upon infusion of ^3H PGE_2 This seems to exclude the possibility that SRS is a metabolic product of PGE_2 Furthermore SRS is not inactivated by incubation with 15-hydroxy prostaglandin dehydrogenase and NAD which also speaks against a prostaglandin nature of SRS (Strandberg and Uvnäs 1971)

Many processes such as nerve stimulation (Ramwell and Shaw 1963 1966 Coceani and Wolfe 1965 Coceani *et al* 1967) inflammation (Willis 1969 Änggård and Jonsson to be published anaphylactic shock (Piper and Vane 1969) administration of biogenic amines e g serotonin (Ramwell Shaw and Jessup 1966 Holmes 1968 acetylcholine Ramwell Shaw and Kucharski 1965 Ramwell *et al* 1966 and catecholamines Shaw and Ramwell 1968 glucagon (Ramwell 1969) ischemia Edwards Strong and Hunt 1969 McGiff *et al* 1969) mechanical stimulation (Edwards Berry and Wyllie 1969 Gilmore Vane and Wyllie 1969) stimulate the formation of prostaglandins Our demonstration that a specific histamine liberator compound 48/80 causes efflux of prostaglandin E_2 in the perfused cat paw is in accordance with the view (Gilmore Vane and Wyllie 1969) that alterations of membrane function is a common feature in situations where prostaglandin biosynthesis is stimulated

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From the Department of Physiology, University of Lund Sweden

Effects of Parasympathomimetic Agents and Vagal Stimulation on the Flow in the Pancreatic Duct of the Cat

By

SVEN LENNINGER

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Abstract

LENNINGER, S *Effects of parasympathomimetic agents and vagal stimulation on the flow in the pancreatic duct of the cat* Acta physiol scand 1971 *82* 345—353

Intravenous injection of methacholine and vagal stimulation increased the amount of juice secreted by the secretin stimulated pancreas of the cat. The increase was greater at low rates

Stimulation of the vagus nerve causes the pancreatic gland to secrete Generally, this secretion does not appear immediately at the onset of stimulation but starts after a few min delay (see Babkin 1928, Hickson 1970, Lenninger and Ohlin 1970) This delay has been attributed to inhibitory vagal fibres (Popielski 1896), contraction of the pancreatic duct (von Anrep 1916, Korovitsky 1923 Scratcherd 1969) and influence of gastric and intestinal movements (Gayet and Guillaumie 1933) In order to further study the restriction to the flow through the pancreatic duct during cholinergic stimulation, the effect of parasympathomimetic drugs and vagal stimulation has been tested in cats on the rate of flow of a secretininduced secretion as well as on the rate by which a salt solution under constant pressure flows through the duct The rate of perfusion has also been studied before and after the injection of secretin

Methods

attached to a 20 cm long polyethylene tubing with inner diameter of 0 86 mm. In the perfusion experiments the duct of the tail of the pancreas was cannulated with a smaller polyethylene tubing (inner diameter 0 58 mm) through which perfusion fluid was led into the gland from a reservoir bottle The fluid left the gland by the tube of the main duct or in some experiments by another polyethylene tubing (inner diameter 0 58 mm) inserted in the

duct of the duodenal part of the gland In the latter case the main duct was ligated In all animals the pylorus was occluded by a ligature and the stomach was drained through an oesophageal tube

In the secretion experiments secretin (GIH Research Unit, Chemistry Department, Karolinska Institutet
by an infusion p
secretin was usua
was studied By
ranging between 1—11 drops/min

In the perfusion experiments the ducts were perfused with a solution containing sodium bicarbonate (144 meq/l) and sodium chloride (25 meq/l)

Methacholine (acetyl β methyl-choline chloride), dissolved in 0 9 % NaCl was injected into one of the femoral veins In the perfusion experiments it was also given directly into the pancreatic duct by injecting the solution into the tube connecting the supply bottle and the gland Acetylcholine (acetylcholine chloride, dissolved in 0 9 % NaCl) was likewise injected intraductally

Vagal fibres were stimulated at the lower end of the oesophagus after the thorax had been opened in the 8th left interspace and artificial respiration by a pump had been arranged The nerves were divided and the distal stumps of the branches merging into the posterior trunk were threaded together through ring electrodes Square wave pulses of supramaximal strength (5—20 V) a duration of 1.5 msec and a frequency of 5—20/sec were given by a Grass stimulator

The pancreatic effluent, whether secretion or perfusion fluid was registered dropwise on a smoked drum by an ordinate recorder which was operated manually or by a photo cell By this means a continuous registration of the drop rate was obtained In most of the experiments the drops were also collected in a test tube to allow direct measurement of the secreted volume
as 0209±
ere was no
> 2 mg/ml)

The protein content of the pancreatic effluent was determined by measuring its light absorption at 280 mμ in a Zeiss spectrophotometer after suitable dilution and comparing the results with a standard solution of bovine serum albumin.

Results

The effects of intravenously injected methacholine and vagal stimulation on the pancreatic secretion

Methacholine was injected in a series of doses 0 01 0 02, 0 05, 0 1 and 1 0 μg/kg to 11 cats in which secretin was infused at various rates It was found to cause an increase in the rate of flow of pancreatic juice in all the animals In two of them the increase was slow and late in onset but in the others the rate increased almost immediately after the injection Only occasionally the first drop after the injection was delayed a few seconds The increase in rate of secretion lasted 2—3 min after which the rate returned to its original level A typical experiment is shown in Fig 1 The increase in rate was positively correlated to the dose of methacholine This was evident from the tracings (Fig 1 and by calculating the increase in number of drops after the various doses of methacholine the correlation was found statistically significant ($P<0.05$) (Fig 2) The pattern of response to methacholine was the same at low and high rates of secretion but the increase was most pronounced when the rate of the secretin induced secretion was low The augmentation of the response to methacholine with decreasing rate of secretion is not only relative but also absolute In Fig 3 the relation between the infused dose of secretin and the increase in secretion is shown



Fig 1 Cat 2 8 kg Chloralose anesthesia Effect of i.v. injections of methacholine (µg/kg) on pancreatic secretion induced by infusion of secretin (0 01 U/kg min) Recordings from above Time in min, drops of pancreatic juice, signal, drop rate Scale left of panel denotes drops/min

The posterior vagus trunk was stimulated in 7 cats Only one rate of infusion of secretin was used for each cat The pattern of response was similar to that seen after the injection of methacholine, *i e* an increase in rate of secretion, only exceptionally preceded by a short delay of the first one or two drops

The concentration of protein in the pancreatic juice was regularly below 2 mg/ml during the control periods After the injection of methacholine the protein content of

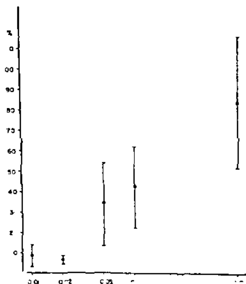


Fig 2 Relation between log dose of methacholine and increase in pancreatic secretion Abscissa the injected dose in µg/kg on a logarithmic scale Ordinate the increase in percent over control The means and S E M are indicated

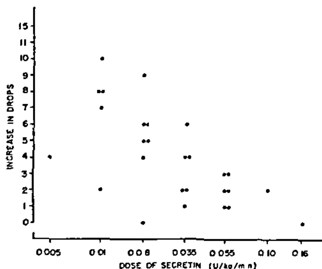


Fig 3 Relation between dose of secretin and increase in number of drops after the i v injection of 1 μg/kg of methacholine in 9 cats

the juice increased in relation to the dose but was unrelated to the rate of infusion of secretin Due to the volume of the draining tube the increase in protein content of the collected juice did not appear until a minute or more had passed since the injection of methacholine The concentration then rose abruptly to values sometimes over 50 mg/ml after the highest dose of methacholine and thereafter declined gradually The protein content of the juice secreted during vagal stimulation increased in most experiments but the concentrations were not as high as after the methacholine injections

The effects of intravenously injected methacholine and vagal stimulation on the rate of perfusion through the pancreatic duct

The pancreatic duct was perfused in 28 cats and the effects of intravenously injected methacholine and vagal stimulation on the rate of flow was investigated The smallest possible hydrostatic pressure was applied to the perfusion fluid to obtain a steady flow through the gland This could sometimes be achieved with the level of the perfusion fluid only 5 cm above the pancreas but in other cases it had to be raised up to 20 cm until a satisfactory flow was ensured Drop rates varying between 5 and 35 drops/min were thus obtained Methacholine was given i v in doses between 0 1 and 2 μg/kg in 19 expts Of 216 injections 156 were followed by a retardation of the perfusion (19 cats), 42 left the perfusion unaffected and after 18 of the injections the perfusion rate increased (9 cats) The retardation was by far the most prominent response (Fig 4) It usually lasted one to two min after which the perfusion retained its original rate In some cases the flow came to a complete stop however, and did not start until a higher pressure was applied to the inflow A graded response to different doses of the methacholine was often seen but it could also happen that the first smaller dose retarded the flow to a greater extent than a following larger dose The responses were



Fig 4 Cat 3 6 kg Chloralose anesthesia. Effect of i v injections of methacholine (μg/kg) on the rate of perfusion of the pancreatic duct Recordings from above time in min signal, drop rate Scale denotes drops/min

also too irregular to allow any conclusions regarding the relation between the perfusion pressure and the degree of retardation The increase in rate of flow seen after 18 of the injections was also of varying magnitude. Sometimes it was very slight but in other cases the drop rate was doubled during the first min after the injection

The posterior vagal nerve was stimulated in 4 cats with 5—10 shocks/sec on 83 occasions during perfusion of the pancreatic duct 45 of the stimulations caused a retardation of the flow, 24 caused no change and 14 stimulations caused the rate of flow to increase Again, the retardations were most prominent and were seen in all 4 cats

The response to methacholine and vagal stimulation was the same whether the perfusion was performed from the duct of the tail to the main duct or to the duct of the duodenal part of the gland Removal of the small intestine below the duodenum did not affect the pancreatic response but often rendered the perfusion more stable Resection of the stomach and the duodenum was performed in 5 animals and in 4 of them a retardation could still be seen after the injection of methacholine

The perfusate was tested for its content of proteins in 10 of the experiments Small amounts were usually present in the perfusate during the control periods After the injection of methacholine and vagal stimulation (Fig 5) the concentration often increased manifold This change in protein content was not invariably found however

Effects of intraductally injected methacholine and acetylcholine on the rate of perfusion through the pancreatic duct

In 11 of the perfusion experiments methacholine and acetylcholine were given intraductally by injecting the agent slowly into the stream of fluid before its entry into the gland The injected volume was 0 1 ml and the doses varied between 0 1 and 10 μg Saline injected in similar amounts did not affect the rate of perfusion Of 130

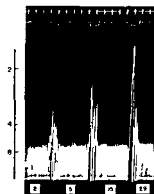


Fig 5

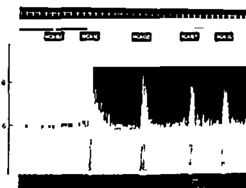


Fig 6

Fig 5 Cat 4 5 kg Chloralose anesthesia Effect of vagal stimulation (3, 5 and 8 shocks/sec 1 5 msec and 8 V) on the rate of perfusion of the pancreatic duct Recordings as in Fig 4 Figures protein concentration in mg/ml

Fig 6 Cat 3 6 kg Chloralose anesthesia Effect of intraductal injections (0 1 ml) of methacholine and acetylcholine on the rate of perfusion of the pancreatic duct Recordings as in Fig 4

injections of acetylcholine and methacholine of various doses 94 were followed by a retardation of the flow of perfusate (Fig 6) while the flow was unaffected after 36 of the injections Again, the responses were not always reproducible and not regularly related to the dose The responses varied from a hardly discernible retardation to a 50 % reduction of flow rate, lasting between 1 to 3 min The rate thereafter returned to its original level The protein concentration of the perfusate was not changed after the injections

The effects of intravenously injected secretion on the rate of perfusion through the pancreatic duct

Secretin in doses varying between 0 2 and 1 0 U was injected i v to 20 cats during ductal perfusion Of 44 injections 11 were followed by an increase in drop rate (7 cats), 24 by a reduction (16 cats) and after 8 of the injections no change in drop

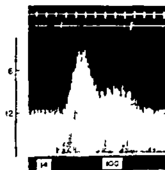


Fig 7 Cat 4 1 kg Chloralose anesthesia Effect of i v injections of 1 clinical unit of secretin at two occasions on the rate of perfusion of the pancreatic duct Recordings as in Fig 4 Figures protein concentration in mg/ml

rate was seen The retardation of flow was usually quite prominent (Fig 7) and lasted 1 to 10 min after which the flow attained its original rate A second injection of secretin, following 5—20 min after an injection that had retarded the flow, was usually without effect on the rate or rather increased it The protein concentration of the effluent was greatly increased, sometimes to more than 100 mg/ml after the first injection of secretin and thereafter declined gradually Subsequent injections of secretin did not further increase the protein content

Discussion

The present experiments show that vagal stimulation and parasympathomimetic agents affect the flow of fluid from the cannulated pancreatic duct of the cat, whether this flow is juice, secreted by the gland or is a salt solution perfused through the duct of the gland The effects of vagal stimulation on the secretion are consistent with the findings of Brown, Harper and Scratcherd (1967), and similar findings have been made in the dog (Thomas 1950) The reports in the literature of the effects of parasympathomimetic drugs on the pancreatic secretion are more conflicting Korovitsky (1923) and Eisler and Ågren (1936) found in the cat that the pancreatic secretion caused by continuous injection of secretin was retarded by pilocarpine In dogs, Lin and Ivy (1957) found that injection of methacholine increased a slow rate of secretion, caused by small doses of secretin but had no effect on a fast secretion, caused by large doses of secretin Continuous infusion of methacholine was not found to affect the response to secretin, and a similar finding was made by Preshaw, Adashek, Cooke and Grossman (1965) In the present tracings the stimulating effect of methacholine on the secretion is clearly seen The increase is of short duration and therefore relatively small and may easily be overlooked if a less sensitive recording technique is used The dropwise recording seemed to be a reliable and suitable method to reveal changes in the secreted volume The finding that the protein concentration of the collected juice increased later than the observed increase in drop rate precludes the possibility of an altered gravity of the drops to cause the change in interval between the drops

The observation that methacholine increased the secretion more when the gland was secreting at a low than at a high rate agrees with the finding of Lin and Ivy (1957) in dogs It may explain why Harper and Vass (1941) did not detect any increase in secretion rate during vagal stimulation since the cats in their experiments secreted at a relatively high rate The present observation may be explained in different ways It may support the assumption of Brown Harper and Scratcherd (1967) that the increase in secretion is secondary to an augmented blood flow and therefore most pronounced when the gland is secreting well below its secretory maximum It may also indicate, however that the cholinergic stimulation apart from promoting the secretion of enzymes causes a watery secretion by acting directly on the same cell as secretin

The vagal stimulation and the injection of methacholine did only exceptionally

cause retardation of the secretion, induced by secretin This retardation may be caused by impeded flow in the duct but may also reflect a decreased production of juice during the transient fall in blood pressure The increased amount of juice secreted in response to cholinergic stimulation was not followed by any compensatory retardation This should be expected if the ducts played an active role in the secretory response as proposed by Korovitsky (1923) and Babkin (1924) The absence of rise in pressure of the pancreatic duct during parasympathetic stimulation (Garrett, Lenninger and Ohlin 1970) does also speak against the theory of an integrated contraction of the pancreatic duct

Contrary to the stimulating effect on the secretin evoked pancreatic secretion the injection of parasympathomimetic drugs and vagal stimulation generally caused the *flow of a salt solution* through the pancreatic duct to be arrested These findings are in agreement with those of Korovitsky (1923) The effects of the stimulation on the perfusion were not as regular as those on the secretion but reproducible enough to confirm that the flow through the duct can indeed be impeded Movements of extra pancreatic structures in response to the stimulation may account for some of the changes in flow rate but the persistence of retardation after removal of the stomach and intestines show that the hindrance to the flow is also to be found in the pancreas itself This was confirmed by the intraductal injections of methacholine and acetylcholine The cause of the retardation may be contraction of smooth muscle cells described by Eberth (1863) to be present in the pancreatic duct of the cat It must also be taken into account however, that the parasympathetic stimulation releases enzymes into the duct and that an ensuing change of fluidity of the ductal content may increase the resistance to the flow That such a factor is *important* for the flow is indicated by the more than occational finding that i v injected secretin caused a marked temporary retardation of the rate of perfusion Secretin is not known to have any smooth muscle effects but on the other hand it is well known that it initially causes a large amount of enzymes to be extruded from the gland This was confirmed in the present perfusion experiments by the high content of protein in the pancreatic effluent after the first dose of secretin

The opposite effects of parasympathomimetic stimulation on the rates of secretion and perfusion may seem contradictory but can be accounted for in the following manner The vagus nerve is secretory to the pancreatic gland of the cat (Lenninger and Ohlin 1969) and when stimulated a small amount of juice is secreted This juice is added to the juice caused by secretin or to the perfusion fluid in the duct When the gland is continuously secreting any increase in resistance is quickly overcome by an increase in pressure within the gland and no retardation of the flow is observed When the duct is perfused at a constant pressure on the other hand the secretory pressure caused by the parasympathetically evoked secretion is too small to overcome the increase in the resistance to the flow and a retardation of the flow follows Thus cholinergic stimulation increases the resistance to the flow in the pancreatic duct and may, under certain experimental conditions prevent or delay the flow of juice from the cannulated end of the duct

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TABLE I Cholesterol and esterified fatty acids mg/100 ml of rat serum after triton and heparin treatment

Treatment		Cholesterol	Esterified fatty acids
Triton	5×25 mg	643±37	6008±653
Triton Heparin	5×25 mg 5×50 IU	533±27	3654±361
Heparin	5×50 IU	111± 1	225+ 6
Saline Controls	5× 0 5 ml	94± 3	223± 7

Method

This study is performed in two different experiments using five month-old male rats of Dawley Sprague strain in both The rats were adopted in laboratory condition for two weeks prior to the experiments They were fed vitaminished and standardised rat feed (Hankkija) and water ad libidum

In *experiment I* the object was to elicit preliminarily the effect of heparin in triton lipemia The rats received the following quantities of Triton WR 1339 (para iso-octyl polyoxyethylene phenol polymer Winthrop Laboratories and heparin (Heparin Medica)

(nine rats)

In exp II the 10 rats in each of the six groups were injected with 3×25 mg of triton at 12 hour intervals and 3×50 3×100 3×150 and 3×250 IU of heparin The controls of this experiment were injected with 3×0 5 of saline All the injections were given i.p in diluted solutions The last injection was administered in the morning at 0800—0900 hours

ht ether
plasma
19ɔ3)

Results

In *exp I* triton caused a sharp increase in the amount of plasma cholesterol and fats Concomitant heparinisation clearly inhibited the increase in cholesterol and above all in the fat concentration (Table I)

In *exp I* triton was administered to the rats for a shorter time and this was probably the reason why the increase in esterified fatty acids and cholesterol in the

TABLE II Esterified fatty acids and cholesterol mg/100 ml of rat plasma after triton and heparin treatment

Triton Heparin	3×2ɔ	3×2ɔ 3 50	3×2ɔ 3×100	3×25 3 150	3×25 mg 3 250 IU	Controls (3×0 5saline)
Esterified fatty acids P <	989±154 0 001	778+1ɔ0 0 01	6?1 81 0 001	464±41 0 001	229+38	233±19
Cholesterol P <	170± 13 0 001	133 13 0 001	128+29	112− 6 0 01	86± 5	7ɔ± 2

rats given triton alone played a smaller but still statistically highly significant role (Table II) Heparinisation inhibited triton lipemia, and the more the heparin was administered With the highest heparin dose used, 250 IU, triton lipemia disappeared completely as regards both cholesterol and fatty acids

Discussion

It has been observed earlier that under the influence of triton some metachromatic material accumulates in the mast cells of the gastrointestinal mucosa and the number of mast cells containing metachromatic material, increases (Haikonen Rasanen and Taskinen 1968) This is perhaps to be interpreted as meaning that triton inhibits the release of the sulphate containing polysaccharide—heparin—from the granules of the mucosal mast cells Triton WR 1339 in dose 3×50 mg caused a 63 per cent increase in the number of mast cells in the rat gastric mucosa, and a 55 per cent increase in the jejunal mucosa The increase in plasma cholesterol was 140 per cent and in esterified fatty acids enormous 2500 per cent However during the triton effect the resorption of fats from the intestine does not change in rat (Kramer and Gioia 1963) and guinea pig (Janicke *et al* 1962) The origin of the lipemia was evidently inhibition of the removal of fat from the circulation

Triton possibly prevents the activation of the clearing factor by inhibiting the liberation of the heparin especially from the mucosal mast cells of the gastrointestinal canal Polysaccharides which reduce the cholesterol level of blood in man activate the clearing factor and inhibit the clinical symptoms of lipemia, have been isolated from human gastric juice and from hog gastric mucosa (Cantone, Rulli and Rossi 1959) Heparin like substance (Smith and Gallop 1953) and heparin synthetising enzyme (Dutton and Stevenson 1959) have been isolated from the dog stomach Heparinoid which possesses the property to activate the clearing factor is found also in the intestinal mucosa of rat duodenum (Bianchini 1958)

Gradually increased heparinisation of rats inhibited triton induced lipemia and when 250 IU of heparin was administered with the triton nullified the lipemic action of triton completely

The increase in plasma cholesterol after long term triton administration (expt I) was 6-fold after the short term treatment (exp II) 2 fold The increases in the fat content of plasma differed more sharply but the antilipemic action of heparin seemed to be equally effective in decreasing the fat as the cholesterol quantities Furthermore compared with the lipemia values of rat plasma in an earlir study of ours (Haikonen *et al* 1968) 3×50 mg of triton caused the same lipemia in rats as in experiment I

Triton obviously inhibits the release of heparin and heparinoids from the mucosal mast cells of the gastrointestinal canal It does not however inhibit the ability of exogenous heparin to activate the clearing factor The effect of growth hormone in producing some degree of lipemia (Winkler *et al* 1964) may perhaps be compared with the triton like effect with metachromatic polysaccharide accumulating also in

the mucosal mast cells of the gastrointestinal canal On the other hand the notable degranulation of mucosal mast cells under the influence of glucocorticoids (Rasanen 1967) or reserpine (Rasänen and Taskinen 1966) changes the fatty acid balance During the effect of glucocorticoid increases the lipemia (Friedman *et al* 1965, Stork, Fabian and Šporanova 1968) while the heparin synthesis of the mucosal mast cells possible declines or is inhited in the gastrointestinal

The mast cell organ of the gastrointestinal mucosa may play a focal role in li polysis already in the resorption phase Clinical observation of cardiosclerosis in hyposecretory persons (Marks, Bank, Krut and Bronte—Stewart 1962) suggests the pos sibility of a paucity of mast cells in the gastric mucosa in which their histamine com ponent functions as a local secretory stimulator (Rasänen 1967) and the secretory capacity is probably influenced by the magnitude of the mucosal mast cell index (Imoto 1969)

This study is sponsored by the aid of Emil Aaltonen s Foundation

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Acta physiol. scand. 1971, 82, 358—374

From the Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden

Purification and Properties of the Slow Reacting Substance Formed in the Cat Paw Perfused with Compound 48/80[1]

By

KJELL STRANDBERG and BORJE UVNÄS

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Abstract

STRANDBERG, K and B UVNÄS *Purification and properties of the slow reacting substance formed in the cat paw perfused with compound 48/80* Acta physiol scand 1971 *82* 358—374

Cat paws were perfused with a physiological salt solution containing *compound* 48/80 In addition to histamine a principle producing a slow sustained contraction of the isolated guinea pig ileum appeared in the effluent This slow reacting substance (SRS) was purified by ethanol extraction solvent partition silicic acid and anion exchange chromatography The overall recovery was 10—20 per cent, calculated from the non histamine smooth muscle stimulating *activity of the ethanolic extract The purification calculated from the crude lipid extract* was about 200 fold The purified material was dialysable thermostable at neutral pH and more labile in acid than in alkaline milieu It behaved homogenously on thin layer chromatography in several systems The most marked biological effects were contraction of the isolated guinea pig ileum and the human bronchus increases in the bronchial resistance in guinea pigs *in vivo in the colouration* (Evans *blue dye* i v) of *guinea pig skin after* intradermal *injection* and in the blood flow in the cat hindlimb after intraarterial injection Treatment of the purified material with N,N-carbo-di p-tolylimide, phenyl isocyanate iodine monobromide potassium *permanganate or acetic anhydride* abolished *or greatly* reduced the biological activity Incubation of SRS with 15 hydroxy prostaglandin dehydrogenase in the presence of NAD resulted in no loss of biological activity Together the results indicate that SRS is a biologically active carboxylic acid with hydroxyl groups and *one* or more double bonds but probably of non prostaglandin nature

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Lipid-soluble smooth muscle stimulating material was shown to appear concomitantly with histamine in the effluents from cat paws perfused with *compound 48/80* (Chakravarty, *Hogberg and Uvnäs* 1959 Since this material elicited a slow sustained contraction *of the isolated guinea pig ileum* it was designated "slow reacting *substance* (SRS) It was later *demonstrated* that this *preparation* consisted of at least two principles with different biological and chemical properties Änggård *et al* 1963)

Recently the less polar principle was identified as prostaglandin E_2 (Änggård and

[1] Part of this investigation was presented at the XIII *Scandinavian Congress* of Physiology Göteborg Sweden Strandberg [illegible]

Strandberg 1971) In the present paper the purification as well as some biological and chemical properties of the principle producing the slow contraction of the guinea pig ileum, the SRS is reported

Experimental procedures and materials

Cat paw perfusion

Solvent extraction and partition

Each lyophilized perfusate was extracted twice with 80 per cent aqueous ethanol 50 ml and

volumes of 1 N NH_4OH which were evaporated to dryness *in vacuo* and stored at $-20°$ C. The ether extracts were washed with water until neutral and evaporated to dryness when determinations of weight and lipid phosphorus were performed All partitions were carried out at 4° C

Column chromatography

Glass columns (1×30—60 cm) equipped with Teflon stopcocks and plugs of glass wool were used The columns were developed either by discontinuous gradient elution or continuous gradient elution. Unless otherwise stated the chromatographies were performed at room temperature with a flow rate of about 2 ml/min All fractions were evaporated to dryness under reduced pressure at room temperature as quickly as possible and when desired the residues were dissolved in Tyrode solution for biological assay All solvents were of reagent grade and except glacial acetic acid redistilled before use

Silicic acid chromatography was performed essentially according to Änggard *et al* (1963) Silicic acid (2—4 g Unisil 100—200 mesh Clarkson Chemical Co Williamsport Pa, USA) was activated at 115° C for one hr prior to use The ether extracts were filtered on to the column while the ammonia-extracted material was applied dropwise after it had been dissolved in methanol and 19 volumes of chloroform had been added. The columns were either developed by stepwise increasing the concentration (v/v) of methanol in chloroform or by a linear

fractionation on DEAE-cellulose.

Cellulose chromatography was performed according to Rouser Galli and Kritchevsky (1965) Whatman standard grade ashless cellulose powder (5 g) was suspended in methanol water (1 1) packed into a column and washed with 65 ml each of methanol water (1 1),

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From the Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden

Purification and Properties of the Slow Reacting Substance Formed in the Cat Paw Perfused with Compound 48/80[1]

By

KJELL STRANDBERG and BORJE UVNÄS

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Abstract

STRANDBERG, K and B UVNÄS *Purification and properties of the slow reacting substance formed in the cat paw perfused with compound 48/80* Acta physiol scand 1971 *82* 358—374

Cat paws were perfused with a physiological salt solution containing compound 48/80 In addition to histamine, a principle producing a slow sustained contraction of the isolated guinea pig ileum appeared in the effluent. This slow reacting substance (SRS) was purified by ethanol extraction, solvent partition, silicic acid and anion exchange chromatography The overall recovery was 10—20 per cent, calculated from the non histamine smooth muscle stimulating activity of the ethanolic extract. The purification calculated from the crude lipid extract was [illegible]out 200-fold The purified material was dialysable thermostable at neutral pH and more [illegible]

material with N,N'-carbo-di p-tolylimide, phenyl isocyanate, iodine monobromide potassium permanganate or acetic anhyd[illegible] Incubation of SRS with 15 hydroxy[illegible]ulted in no loss of biological activity [illegible] active carboxylic acid with hydroxy[illegible] of non prostaglandin nature

Lipid soluble smooth muscle stimulating material was shown to appear concomitantly with histamine in the effluents from cat paws perfused with compound 48/80 (Chakravarty, Hogberg and Uvnas 1959) Since this material elicited a slow sustained contraction of the isolated guinea-pig ileum it was designated "slow reacting substance" (SRS) It was later demonstrated, that this preparation consisted of at least two principles with different biological and chemical properties (Änggård *et al* 1963)

Recently the less polar principle was identified as prostaglandin E_2 (Änggård and

[1] Part of this investigation was presented at the XIII Scandinavian *Congress of Physiology*, Göteborg, Sweden (Strandberg 1969)

Incubation with 15 hydroxy prostaglandin dehydrogenase

Purified SRS in the first experiment 890 U and in the second experiment 1500 U was incubated at 37° C for 30 min in 0 3 ml of 0 1 M Tris buffer pH 8 0 containing 5 mM NAD and 0 15 mU of 15-hydroxy prostaglandin dehydrogenase (PGDH)[1] from swine lung (Ang gård and Samuelsson 1966) Control incubations of the same material were carried out where NAD, PGDH or both were omitted from the incubation medium Directly after the incubation period the SRS activity of the samples was determined on the guinea pig ileum (*vide infra*)

Preparation of crude gangliosides

Brains obtained from cats used for perfusion experiments were homogenized in a Waring blendor and repeatedly extracted with acetone The residues were extracted with methanol chloroform (2 1) (10 ml/g) for 5 hrs in a Soxhlet apparatus The extracts were concentrated by evaporation under reduced pressure dialysed against running tap water (2 days) and distilled water (one day) and lyophilized

Biological assays

Isolated smooth muscle preparations Segments of various organs were suspended in a 4 or 25-ml (human bronchi) organ bath of controlled temperature Except for human bronchi longitudinal movements were recorded isotonically on a smoked drum with a frontal writing leve

T nt $MgCl_2$ 0 02

per t.

L cent $NaHCO_3$

0 05 per cent glucose 0 1 per cent

Krebs Henseleit solution NaCl 0 69 per cent KCl 0 035 per cent $CaCl_2$ 0 028 per cent $MgSO_4$ 0 011 per cent, $NaHCO_3$ 0 21 per cent, KH_2PO_4 0 014 per cent glucose 0 1 per cent.

Human bronchi were dissected out from macroscopically non-diseased lung tissue obtained in connection with surgery for pulmonary carcinoma Other organs were prepared after stunning and bleeding of the animals

Guinea pig ileum Terminal ileum from guinea pigs weighing 200—350 g Tyrode solution air 37° C

Guinea pig colon Proximal colon from animals weighing 300—350 g Tyrode solution air 37° C

Guinea pig uterus Uterine horns from non pregnant guinea pigs 325—350 g in weight. D

Jalon solution O_2 37° C

on solution O_2 18° C

... 160 g De Jalon solution O_2 30° C

Rabbit duodenum Proximal duodenum from animals weighing 3—4 kg Tyrode solution O_2 37° C

Rabbit trachea Spirally cut trachea from animals 3 0—3 5 kg in weight Krebs Henseleit solution, 5 per cent CO_2 in O_2 37° C The effect of agonists *per se* as well as their potency to inhibit acetylcholine induced contractions (Main 1964) were studied

Human bronchus Spirally cut bronchi approx. 3 mm in diameter were connected to a Grass force-displacement transducer for isometric recordings using a Grass polygraph as described by Mathe Strandberg and Åstrom (1971) Tension 0 5 g Tyrode solution 6 5 per cent CO_2 in O_2 37° C

Assay of SRS was performed on the isolated guinea pig ileum (*vide supra*) principally as described by Chakravarty (1959) The T

and mepyramine maleate (1 g/ml)

version of the overflow method

... was unopened A cannula was inserted into the lower trachea and the lungs were inflated using a Braun pump driving a constant stroke volume (8—14 ml) at 72 strokes/min The stroke volume was adjusted at the beginning of each experiment to give a minimal overflow volume The excess air which did not enter the trachea was directed via a side arm of the tracheal cannula to a non return water

[1] The enzyme preparation was kindly supp...

valve set for a pressure of 10 cm of water, connected to a Palmer piston recorder writing on a kymograph drum. An increase in air overflow indicated an increased bronchial resistance. When the peak of the response was reached after intrajugular administration of a bronchoactive compound the recording side arm was clamped for 10 sec., once or twice, to inflate the lungs forcibly.

Vascular effects in skin. Albino guinea-pigs 500—800 g in weight were used. Hair on the dorsal back was removed as completely as possible by the use of an electrical clipper. To avoid traumatically induced changes in the permeability, the technique for preparation and injection described by Miles and Miles (1952) was essentially followed. All solutions for injection were made up in the above mentioned salt solution used for perfusion.

The following procedures were principally adopted from Orange, Stechschulte and Austen (1969). All [illegible] were injected intradermally with 0.1 ml volumes of the test compounds.[1] Immediately after the injections, the subcutaneous veins on the posterior aspects of the thigh were exposed by incision of the skin and 1.0 ml of 1.0 per cent Evan's blue dye was injected. The animals were lightly anesthetized with ether during these steps. Thirty min later, the animals were sacrifized in ether, the skin was reflected and transilluminated. The cross diameter of the lesions and the intensity of "blueing" were determined.

Blood flow and blood pressure. Cats, weighing 3—4 kg, were anesthetized with sodium pentobarb[illegible] [illegible]lated. Heparin (2 [illegible] [illegible] n exteriorized loop in [illegible] Close arterial injectio[illegible] [illegible] pressure was recorded in the left femoral artery by a Statham pressure transducer. A Grass polygraph was used for both recordings.

Results

A. *Purification*

Extraction and partition

An outline of the purification procedure is shown in Fig. 1. In confirmation of earlier results (Chakravarty *et al.* 1959) SRS was completely extracted with 80 per cent aqueous ethanol from a lyophilized perfusate and it passed into ether from an aqueous phase at an acid pH. After three extractions less than 5 per cent of the non-histamine smooth muscle stimulating activity remained in the aqueous phase. The material could not be extracted into a less polar solvent such as petroleum ether. According to its acidic nature SRS passed into phosphate buffer (pH 8.0) from the ether phase. The recovery of the non histamine smooth muscle stimulating material from the ethanolic extract was 67 ± 10 (M $\pm$ SE, n = 6). The active principle was taken up into ether at pH 3 prior to subsequent silicic acid chromatography.

Silicic acid chromatography

When the combined ether extracts were applied to a silicic acid column all SRS activity was adsorbed. The column was fractionally eluted with increasing amounts of methanol in chloroform (Fig. 2). Most of the spasmogenic material was eluted with methanol: chloroform (3:7). Smaller amounts appeared in the methanol: chloroform (1:1) fractions as well as in the final methanol eluates. In seven experiments the silicic acid column was eluted with a linear gradient of methanol in

[1] Prostaglandins were kindly given by Prof. S. Bergström, Department of Chemistry, Karolinska Institutet, Stockholm, Sweden. Synthetic bradykinin (batch 67236) was generously supplied by AB Sandoz, Stockholm, Sweden.

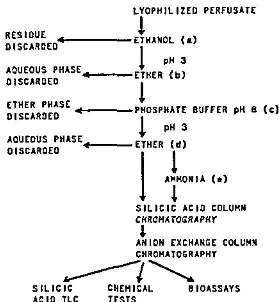


Fig 1 Outline of procedures for purification and characterization of SRS When non pooled effluents were processed steps b and c were usually omitted

chloroform starting with 5 per cent and ending with 100 per cent of methanol When the fractions were tested on the guinea pig ileum only one peak of activity was found This indicated that the earlier observed fractionation of the biological activity was due to incomplete elution In large scale work ups SRS was generally eluted with methanol chloroform (1 1) after methanol chloroform (1 9)

The polarity of SRS resembled that of a phospholipid Determination of phosphorus in the different fractions revealed that phosphorus was present in biologically active as well as in other fractions (Fig 2)

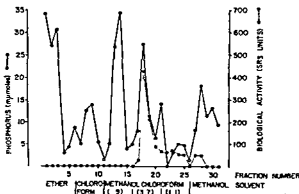


Fig 2 Silicic acid chromatography of lipid soluble smooth muscle stimulating material extracted from pooled effluents from 4 cats Column 4 g Fraction volume 10 ml except the first fraction which constituted the sample volume

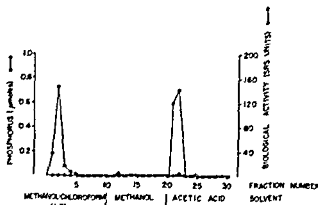


Fig 3 Separation of biological activity from material containing phosphorus by anion exchange chromatography on Ecteola cellulose Column 2 g Fraction volume 5 ml

Anion exchange chromatography

The SRS obtained by silicic acid chromatography was dissolved in methanol chloroform (1 7) and applied on a column prepared of Ecteola cellulose The spasmogenic principle was retained by the column whereas nearly all the phosphorus containing material passed through (Fig 3) Methanol was ineffective in achieving elution of SRS while organic solvents containing acetic acid displaced the active material from the column Also here only one peak of activity was found when the column was eluted with a linear gradient of acetic acid in chloroform starting with 100 per cent chloroform and ending with 100 per cent acetic acid Acetic acid was routinely used to achieve complete elution

Recovery

Considerable losses of smooth muscle stimulating activity were encountered during the purification procedures The overall recovery was usually 10—20 per cent calculated from the non-histamine biological activity of the ethanolic extract The lost SRS could not be recovered from other fractions The low yield of active material in the final stage is therefore probably due to chemical inactivation or loss of possible potentiating effect of separated factors The results from a representative experiment where the pooled effluents from paws of four cats were processed are shown in Table I It is seen that in this experiment most of the activity was lost during the

TABLE I Purification of SRS appearing in the effluents from cat paws perfused with compound 48/80 The figures given for the purification refer to the steps after the ethanol and ether extractions

Procedure	Weight (mg)	Biological activity (U)	Purification	Yield (%)
Ethanol extraction	4910 00	44,300		100
Ether extraction	4 60	20 250		46
Silic acid chromatography	0 09	8,880	22	20
Ecteola chromatography	~0 01	8 190	~186	18

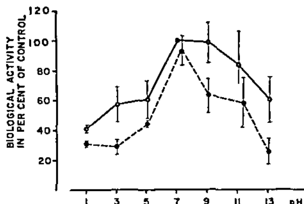


Fig 4 Influence of heat and pH on the biological activity of SRS dissolved in salt solution The pH values were adjusted by the addition of small volumes of 1—6 N HCl or 1—3 N NaOH Bioassay was performed after neutralization of the samples Open symbols represent samples heated in a boiling water bath for 15 min, filled symbols denote iced controls The results are expressed as percentages of the biological activity of the iced sample, pH 7 0 Means and ranges of 2 expts

extraction and silicic acid chromatography whereas the recovery from the Ecteola column was quantitative Essentially similar results were obtained in other experiments

The final material contained 8,190 U weighing about 0 01 mg Thus 1 U, representing an average threshold dose would be equivalent to about 1 ng of the purified material

The smooth muscle stimulating material obtained after anion exchange chromatography was examined as follows

B *Chemical properties and chromatographic behaviour*

General properties

SRS was dialysable Thus when the purified material was dissolved in 10 ml of isotonic salt solution, pH 7 0, and subjected to dialysis[1] against 11 ml of the same solution for 16 hrs at 4° C 23 per cent of the recovered biological activity was in the outside fluid The salt concentration as well as binding to the membrane may have influenced the result

In the dry state (acid form) SRS was completely soluble in methanol but practically insoluble in ether chloroform benzene and petroleum ether

Heating of the spasmogenic material for 15 min at 100° C and pH 7 0 resulted in no loss of biological activity as compared to the activity of a control sample kept at 4° C The stability decreased at both sides of neutrality more in acid than in alkaline milieu, heating intensified the influence of pH (Fig 4)

The stability also decreased at storage (−20° C), here the purified material was dissolved in salt solution Thus about 60 per cent of the biological activity was lost after storage for 2 weeks and after 4 weeks only about 10 per cent of the activity remained

The distribution of the spasmogenic material in a two-phase system consisting of

[1] Union Carbide Corp no 18/32 24 mm flat

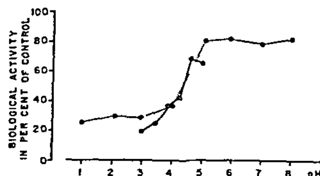


Fig 5 Distribution of SRS between equal volumes (2 ml) of ether and salt solution at different pH values adjusted by the addition of small volumes of HCl or NaOH The samples were agitated and centrifuged (4° C) The aqueous phases were neutralized and bioassayed. The results are expressed as percentages of the biological activity of a non partitioned control, pH 7 0 Means of duplicate samples, 2 expts

equal volumes of ether and salt solution at pH values ranging from 1 0 to 8 0 was compatible with that of a carboxylic acid The results are illustrated in Fig 5 Virtually all recovered biological activity was in the aqueous phase at pH 5 1 and above Progressively lesser amounts of SRS were recovered from the aqueous phase by lowering the pH-value

Tests for functional groups

For full biological activity the spasmogenic material appears to be dependent on intact hydroxylic and carboxylic groups as well as on unsaturated bond(s (Table II) It is true that phenyl isocyanate and acetic anhydride not only react with hydroxylic groups but also with amino groups However, since the acidic nature of SRS is clearly established the presence of amino groups in SRS does not seem likely

Incubation with 15 hydroxy prostaglandin dehydrogenase

15 Hydroxy prostaglandin dehydrogenase isolated from swine lung is specific for the 15 (s) hydroxy groups in the prostaglandins (Änggård and Samuelsson 1966 Nakano Änggård and Samuelsson 1969) The biological activity of the resulting 15-keto metabolites is low compared to that of the parent compounds (Änggård 1966, Pike, Kupiecki and Weeks 1967) When SRS in two experiments was incubated with prostaglandin dehydrogenase in the presence of NAD, no biological inactivation occurred. This indicates that SRS is probably not of prostaglandin nature

Table II Influence of chemical reagents on the biological activity of SRS The results are expressed as percentages of the biological activity of controls Each value represents 1 expt. NM = non measurable

Reagent	Functional group	Biological activity in per cent of control	
Phenyl isocyanate	—OH	NM	NM
Acetic anhydride pyridine (1 1)	—OH	NM	NM
N,N'-carbo-di p-tolylimide	—COOH	NM	30
Iodine monobromide	—CH=CH—	NM	NM
Potassium permanganate	—CH—CH—	NM	NM

TABLE III Thin layer chromatography of SRS Each Rf value represents the mean of 2—5 expts, ranges are given within brackets

Solvent system (composition in ml)	Rf value
I Chloroform methanol acetic acid water (40 20 5 3)	0 55 (0 45—0 63)
II Chloroform methanol water (75 25 4 2)	0 11 (0 10—0 11)
III Chloroform methanol water (60 35 8)	0 31 (0 23—0 35)
IV Propanol water (3 1)	0 38 (0 32—0 43)
V Propanol ammonia water (6 3 1)	0 59 (0 54—0 63)

Thin layer chromatography

The spasmogenic material behaved homogenously in all the solvent systems used, *i e* no fractionation of the biological activity occurred (Table III) The recovery was 15—45 per cent

Cellulose chromatography

Smith (1966) stated that antigen induced SRS from guinea pig lung is a ganglioside Since gangliosides can be selectively separated from other lipid classes by chromatography on cellulose (Rouser *et al* 1965) the behaviour of the present SRS was studied in such chromatography Crude gangliosides (10 mg—71 μg N acetyl neuraminic acid) from cat brain were used as reference since the neuraminic acid content of the purified spasmogenic material (1000 U) was non measurable In two experiments no biological activity was retained by the column wheras nearly all material 94 and 96 per cent respectively, which gave a positive reaction for neuraminic acid was retained A dissociation of the two activities was also noted when Ecteola chromatography was performed Thus fractions eluted with acetic acid chloroform (1 3) contained only spasmogenic activity 100 per cent acetic acid was needed to elute neuraminic acid containing material

C *Biological effects*

Effect on isolated smooth muscle organs

The effect of purified SRS on different smooth muscle organs from various species was investigated and related to the effect of prostaglandin E_1 (PGE_1) or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) The results are presented in Table IV It is seen that SRS, 100 U/ml produced no response in five of the nine types of preparations studied In contrast PGE_1 where used produced contractions at about the same dose level in all but one of the preparations The guinea pig ileum and the human bronchus were most sensitive to SRS For both these organs there was a latency period of 10—30 sec and about 1 min respectively and the effect was dose-dependent The contractions were slow 1—3 min and 4—10 min respectively were required before the peak contraction was reached The contractions were sustained and slow relaxation

TABLE IV. Threshold doses on various smooth muscle preparations for SRS and comparison of activity with PGE_1 or $PGF_{2\alpha}$. The means of 3—4 expts are presented, ranges are given within brackets More than 100 U of SRS per ml bath fluid was never administered

reparation	Threshold dose per ml bath fluid		
	SRS (U)	PGE_1 (ng)	$PGF_{2\alpha}$ (μg)
Guinea pig ileum	0 8 (0 4—1 6)	33 (25—50)	
Guinea pig colon	29 (12—50)	5 1 (3 0—6 3)	
Guinea-pig uterus	> 100	5 0[1]	
Rat colon	58 (50—75)	3 1 (0 5—6 3)	
Rat uterus	> 100	1 8 (0 5—2.5)	
Hamster colon	> 100	10 (6—13)	
Habbit duodenum	> 100	5 0 (2 5—6 3)	
Rabbit trachea	> 100[2],[3]	150 (100—250)[3]	
Ruman bronchus	4 1 (2 0—6 2)		< 0 4,[4] < 1 25[4]

[1] the threshold dose was the same in all three expts
[2] contraction or relaxation,
[3] inhibition of acetylcholine induced contractions
[4] definite threshold dose was not established, each value was obtained in two different expts

lowed on washout The effect of histamine and bradykinin on the guinea-pig ileum was enhanced by a preceding administration of SRS Fig 6 illustrates the effect of SRS on isolated human bronchi

ffect on bronchial resistance

e action of SRS on the bronchial resistance in guinea-pigs was studied in six experiments using a modified version of the Konzett-Rossler (1940) overflow method The mean threshold dose for response was found to be about 1100 U (range 500—1800 U) This is an approximate figure since a true determination was invalidated by tachyphylaxis With this limitation the threshold dose was equivalent to 0 25—0 50 μg of histamine

Vascular effects in guinea-pig skin

Orange *et al* (1969) reported that intradermally injected SRS-A[rat] produced an increase in the capillary permeability in guinea-pig skin as measured by the subsequent distribution of intravenously administered Evan's blue dye The same technique was used in the present experiments to evaluate the effect of purified cat paw SRS

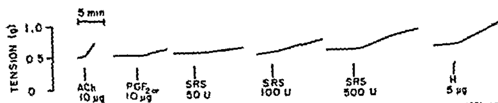


Fig 6 Effect of SRS, acetylcholine (ACh), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and histamine (H) on an isolated human bronch s 25-ml bath, Tyrode solution, 6 5 per cent CO_2 in O_2, 37° C

TABLE [illegible]

Compound	Concentration per site	Diameter of lesion	Intensity of colouring
Control (13)		2 × 3 (0—3 × 0—4)	+
SRS	100 U (7)	9 × 12 (5—12 × 11—15)	+++
	25 U (5)	5 × 6 (3—9 × 5—9)	+
PGE_1	100 ng (7)	5 × 7 (3—9 × 4—11)	++
	25 ng (5)	5 × 6 (3—8 × 5—9)	++
Bradykinin	100 ng (5)	4 × 6 (3—8 × 3—9)	++

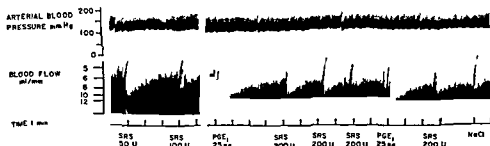


Fig 7 Effect of SRS and prostaglandin E_1 (PGE_1) on the blood flow in a cat hindlimb preparation Cat 3 2 kg, anesthetized with sodium pentobarbital, 25 mg/kg i.v

The results are summarized in Table V It can be seen that 25 U of SRS produced about the same response as 25 ng of PGE_1 and 100 ng of bradykinin However, when the concentration of SRS and PGE_1 was increased 4-fold, SRS produced a somewhat greater response than PGE_1

Effect on blood flow and blood pressure

The effect of SRS on the blood flow in the cat hindlimb was studied in 3 experiments One of the experiments is shown in Fig 7 Here it is seen that a close arterial injection of 50 U of SRS produced an immediate but transient increase in blood flow without influencing the systemic blood pressure On repeated administration of SRS the response was less pronounced Tachyphylaxis also occurred in one of the two other experiments Therefore no clearcut relationship between the potency of SRS and PGE_1 could be worked out However, the first injection of 50 U of SRS always produced a significant response yet this was smaller than that produced by 10 ng of PGE_1

Discussion

In the present study the principle in the effluents from cat paws perfused with compound 48/80, which elicits a slow, sustained contraction of the isolated guinea pig ileum (SRS response) has been purified by ethanol extraction, solvent partition, silicic acid and anion exchange chromatography. The purified spasmogenic material behaved homogenously on thin layer chromatography in several systems. It is an acidic, polar and dialysable compound and resists boiling at neutral pH. The biological activity decreases at storage and by treatment with hydrochloric acid. These findings confirm and extend earlier observations on the chemical properties of this spasmogenic principle (Chakravarty *et al.* 1959, Änggård *et al.* 1963).

The purified material seems to have a limited range of spasmogenic activity, as judged from the tests on various smooth muscle organs. Thus the guinea pig ileum and the human bronchus were noted for their pronounced reactivity to SRS. Here, the threshold dose was as low as about 1 ng/ml bath fluid. The potent bronchoconstricting action of our SRS was particularly interesting to note in view of the findings that SRS-A (SRS of anaphylaxis) is released from lungs of asthmatic patients challenged *in vitro* with specific allergen (Brocklehurst 1955) and in passive anaphylaxis *in vitro* using human lungs sensitized with reaginic serum (Sheard, Killingback and Blair 1967). Bronchoconstricting action of SRS-A from anaphylaxis in guinea pig lung (Brocklehurst 1955, Berry and Collier 1964) has been demonstrated earlier. However, the SRS preparation used in these studies was rather impure, mainly histamine free. This is of significance in light of recent findings that prosta[illegible]ins are released during anaphylactic reaction in guinea pig lung (Piper and [illegible]e 1969) and their effect on the tracheobronchial tree (for references see Berg[illegible]tröm, Carlsson and Weeks 1968). The more detailed study of the bronchoconstricting action of our purified material will be reported in a separate communication (Mathe and Strandberg 1971).

The purified material was found to increase the bronchial resistance in guinea pigs *in vivo*. Here, in contrast to the effect on isolated human bronchi, tachyphylaxis occurred, as has also been demonstrated earlier for SRS-A (Berry and Collier 1964). This might indicate that in the guinea pig the action on the bronchi was mediated via the release or activation of some other factor.

Intradermally injected SRS produced a local increase in the colouration of guinea pig skin upon i.v. injection of Evans blue dye. Whether this was due to an increase in the porosity of the capillary wall and/or a vasodilatation cannot be evaluated by the technique used. However, in support for the latter alternative is the finding that SRS produced an increase in the blood flow in the cat hindlimb. An increase in the capillary surface area, due to a dilatating effect of SRS, is compatible with the present observations. Similar effects (*cf.* Miles and Miles 1952, Spector and Willoughby 1968, Juhlin and Michaelsson 1969, Fredholm, Öberg and Rosell 1970) are produced also by the other two substances known to be released in the cat paw by compound 48/80, *i.e.* histamine and prostaglandin E_2. These vascular effects are of interest since activation of mast cell release mechanisms has been proposed as one

explanation for the development of the inflammatory response in injury (for references see Spector and Willoughby 1968) Furthermore the present results indicate a similarity between the present SRS (cat paw) and SRS-A[rat] (Orange *et al* 1969)

Feldberg and Kellaway (1938) introduced the designation "slowly contracting substance" to denote a non histamine smooth muscle stimulating principle appearing in the effluents from some animal tissues perfused with cobra venom Since this term is merely a generic description referring to a mode of biological action, spasmogenic principles detected under other experimental conditions have also been designated "slow contracting substance" or 'slow reacting substance", *e g* in anaphylaxis in guinea pig lung (Kellaway and Trethewie 1940, Brocklehurst 1960), after the action of snake venom on egg yolk (Vogt 1957), in cat plasma after intravenous injection of compound 48/80 (Paton 1951) or thalassine (Jaques and Schachter 1954), *in the effluents from cat paws perfused with compound 48/80* (Chakravarty *et al* 1959, Änggård *et al* 1963, Strandberg 1971) and in passive anaphylaxis in the rat (see Austen, Orange and Valentine 1968)

However, it seems as a variety of spasmogenic substances are denoted by SRS Thus SRS from egg yolk was reported to consist of certain unsaturated fatty acids (Vogt 1957) Änggård *et al* (1963) showed that, regardless of source, SRS formed in guinea pig lung (antigen), cat paw (compound 48/80) and rat peritoneal mast cells (compound 48/80) consisted of at least two components Recently prostaglandins have been found to be part of venom induced SRS (SRS-C) (Vogt *et al* 1969) and SRS A (Piper and Vane 1969) SRS appearing on anaphylactic histamine release in guinea pig lung tissue has been claimed to be a ganglioside (Smith 1966) However the SRS principle purified in the present work, although quite hydrophilic, does not seem to be a ganglioside since it behaved as one neither in cellulose chromatography nor in anion exchange chromatography

In the present study the amounts of purified material were small, which hampered structural elucidation Direct inlet mass spectrometry and combined gas liquid chromatography and mass spectrometry after treating the purified material with diazomethane and acetylation were tried, but in vain To get some information of the structure an indirect approach was employed Thus the material was treated with chemical agents known to react with certain functional groups This technique has previously been used successfully as a supplement to other methods for identification of minute quantities of biologically active principles (*cf* Ambache 1959 Dakhil and Vogt 1962, Ramwell and Shaw 1966) Judging from the results of the present study the purified smooth muscle stimulating principle is an unsaturated hydroxycarboxylic acid

Ambache (1966) stated that all biologically active lipid soluble principles had proved to be unsaturated acids carboxylic or phosphatidic with one or more hydroxylic groups To our knowledge this statement has not been contradicted by later studies Out of these two classes of lipid acidic principles carboxylic acids alone, and mainly prostaglandins have actually been reported to be released from tissues (for references see Bergstrom *et al* 1968 Ramwell 1969) The present [illegible]

principle is not identical with any known prostaglandin Neither does a prostaglandin nature of the compound seem likely since prostaglandin dehydrogenase had no effect on its biological activity However, there are some indications that prostaglandins and SRS may be the products of common or related processes Thus it has been shown that PGE_2 and SRS constitute the main part of lipid-soluble spasmogenic material appearing in effluents from cat paws perfused with compound 48/80 (Änggård and Strandberg 1971) Anaphylactic histamine release from sensitized guinea pig lung tissue as well as histamine release from isolated rat peritoneal mast cells caused by compound 48/80 is accompanied by the appearance of spamogenic principles with prostaglandin and SRS like properties (Änggård *et al* 1963) Incubation of guinea pig lung (Fredholm and Strandberg 1969) and cat lung tissue (Ladinsky and Strandberg 1969) with phospholipase A lead to the appearance of smooth muscle stimulating principles with similar properties

In this context it seems relevant to recall that Hogberg and Uvnas (1957 1960) suggested that degranulation of mast cells and release of histamine entail the activation of a phospholipase A or a related lipolytic enzyme localized to the mast cell membrane In support for this view were the observations made with the Cartesian diver technique on isolated mast cells which showed that such cells harbour a factor that splits lecithin but not lysolecithin (Giacobini Sedvall and Uvnas 1965)

Prostaglandins are similarly to SRS considered to result from membrane processes activated in connection with secretory and release phenomena (see Bergstrom *et al* 1968 Ramwell 1969) They have been suggested to be biosynthetized from fatty acids resulting from phospholipase A induced hydrolysis of membrane phospholipids Vogt Suzuki and Babilli 1966 Coceani *et al* 1967) In case of a common origin—membrane phospholipids—and a common generator—phospholipase A—of prostaglandins and the SRS-principle one might expect them to occur together They do so on histamine release due to mast cell degranulation *i e* a cell response involving active cell membrane processes leading to expulsion of amine carrying granules It might be rewarding to look for the appearance of SRS in other secretory and release processes where prostaglandins have been observed to occur

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Acta physiol. scand. 1971. 82. 375—381
From the Institute of Physiology, University of Oslo, Norway

Hyperosmolarity and Pulmonary Vascular Capacitance

By

G Bo, A Hauge and G Nicolaysen

Received 15 January 1971

Abstract

Bo, G, A Hauge and G Nicolaysen *Hyperosmolarity and Pulmonary Vascular Capacitance* Acta physiol scand 1971 82 375—381

Since plasma osmolarity rises during general muscle exercise and the lung is thought to have a function as a blood depot we have investigated the effect of plasma hyperosmolarity on pulmonary vascular capacitance Hyperosmolar solutions of sodium chloride, urea and ethylene-

This work has been motivated by considerations related to 3 sets of experimental findings

1) The lung appears to have a function as a blood depot which can be drawn upon during various forms of circulatory stress, *e g* acute hemorrhage (Aarseth 1970)

2) Elevation of extracellular osmolarity in a rat portal vein preparation will induce a fall in smooth muscle cell volume and a concomitant volume-related inhibition of electrical and mechanical activity of the smooth muscles (Johansson and Jonsson 1968) Furthermore, resistance vessels of the lung relax when exposed to moderate elevation of blood osmolarity (Hauge and Bo 1971)

3) The osmolarity of the effluent blood of exercising muscles is increased. Systemic arterial osmolarity has been found to be elevated by 25 mosm/l after a few min of general exercise (Lundvall *et al* 1970) The osmolarity of mixed venous blood must then be elevated even more

The two last observations first led us to wonder whether the capacitance vessels of the lung would also relax during general exercise Such a response seemed, however to be teleologically disadvantageous as it would give a tendency towards pooling of blood in the lung If the lung has a function as a blood depot we would rather expect a reduction of pulmonary blood volume during exercise We therefore deci

investigate the response pattern of the pulmonary capacitance vessels during slow, graded rises in plasma osmolarity

A preliminary report on some of the data has been given elsewhere (Bø, Hauge and Nicolaysen 1970)

Methods

The lung preparations were taken from albino rabbits of either sex weighing 2870 ± 230 g The rabbits were anesthetized with pentobarbitone (Nembutal® Abbott) 30—50 mg/kg i v Heparin —750 I U /kg—was also given i v The procedure for removal of the heart and lungs and for connection of the lungs to the perfusion circuit was as described by Hauge Lunde and Waaler (1965)

Perfusion of the pulmonary vascular bed was carried out with a Dale & Schuster pump at constant volume pulsatile inflow The perfusate was heparinized (3000 I U /100 ml) horse plasma which was kept frozen until 1 hr before the start of an experiment It was then thawed at 38° C and filtered through one layer of filter paper In all the experiments a flow of 250 (± 5) ml/min was selected and the perfusion was started 10 to 12 min after the rabbit's own circulation had been stopped. The first 15 ml of effluent perfusate were discarded in order to bring the hematocrit down to well below 1 per cent The circulating perfusate volume was 210 (± 2) ml at the outset of each experiment. The outflow pressure (= left atrial pressure, P_{LA}) was kept constant in each experiment and at 0 5—1 5 cm of water The inflow pressure, P_{PA} was recorded with a Statham P23Db pressure transducer connected to a Sanborn, two-channel model 320 recorder Perfusate temperature was kept at 38° C

Ventilation was carried out using a Starling 'Ideal' pump (C F Palmer London) and positive pressure technique End tidal pressures were kept at 10 and 1 5 cm H_2O respectively by the use of water seals Ventilation gas was 5 % CO_2 in air Ventilation overflow was measured by the method of Konzett and Rossler (1940)

Weighing The preparation was suspended under a Sanborn force transducer (FTA 100 1) by the use of a string which was fastened to a string around the atrioventricular groove Since the axis of the transducer had a very low displacement on variations in load only diminutive changes in tension of the perfusion and ventilation tubings could occur when changes in preparation weight developed Calibration of the system was done at the end of every experiment by placing different known weight loads on the preparation Weight changes down to mg could be detected and recorded on the Sanborn recorder

Test solutions were infused into the pulmonary arterial tubing from syringes mounted in a flow infusion pump (Harvard model 947) Different stepwise elevations of plasma osmolarity were obtained by changing the speed of the infusion pump Three test substances of different chemical nature and with different membrane permeability characteristics were chosen sodium chloride urea and ethyleneglycol The osmolarity of the test solutions were measured in 1 20 dilution with distilled water by the use of a Knauer osmometer The osmolarity of the undiluted test solutions are listed below

Sodium chloride solution	7000 mosm/l
Urea solution	5680
Ethyleneglycol	8600

Results

The start of the perfusion and the ventilation caused an immediate rise in the weight of the preparation This increase in weight was interpreted as being due to an increase in the intravascular volume of the lungs After such an initial adjustment the weight of an undisturbed preparation remains stable or with small and slow adjustments for at least two hr thus allowing tests with hyperosmolar solutions

Fig 1 demonstrates an example of a response to a step increase in plasma osmolarity The test solution of sodium chloride was infused for a period of 45 sec A period of this length was chosen in order to avoid recirculation of test solution during the infusion. The hyperosmolar solution caused a reduction of the inflow pressure and

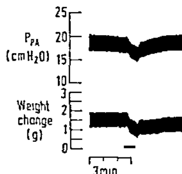


Fig 1 *Effects of an elevation of plasma osmolarity of 43 mosm/l* Isolated perfused rabbit lungs Flow 250 ml/min test solution sodium chloride 7000 mosm/l, infusion rate 1.53 ml/min infusion period 45 sec P_{PA} pulmonary arterial pressure

at the same time, a fall in the weight of the preparation of about 1 g The total weight of the lung preparation is about 18 g, of which at least 11 g will be intravascular fluid (Lunde 1967) Thus, if the total weight reduction was due to a shift in perfusate volume from lungs to the extrapulmonary part of the perfusion circuit, a ten per cent reduction in vascular capacitance would have taken place Reduction in inflow pressure was regularly seen as a response to the first infusion but, in most cases could not be obtained upon repeated infusions in one and the same pair of lungs The weight responses were, however, always transient and reproducible No change in ventilation overflow was seen as response to this or other infusions of hyperosmolar solutions

Our first object was then to obtain knowledge about the dose/response relationship between stepwise rises in plasma osmolarity and weight reductions of the lungs Nine lung perfusions were carried out in order to examine the effect of each of the 3 test substances, *i e* 3 perfusions per test substance were performed An infusion period of 45 sec was selected for all the 27 infusions The individual dose/response curves were constructed on the basis of 3 stepwise elevations of plasma osmolarity Before the start of the first infusion a plasma sample was collected and plasma osmolarity determined For the stepwise elevations of plasma osmolarity we used calculated values, knowing the osmolarity of the test solutions, the infusion rate and the plasma flow rate

The results are listed in Table I and a graphical representation is demonstrated in Fig 2 Weight reductions obtained after 45 sec of infusion are plotted against the induced osmolarity gradients defined as the increase in perfusate osmolarity during

TABLE I Reductions in lung weight in g caused by rises in plasma osmolarity Each figure represents the mean values from three expts

Increase in osmolarity / Osmolarity at start of infusion	NaCl	Urea	Ethyleneglycol
0 083	0 23	0 27	0 07
0 166	0 56	0 48	0 26
0 25	0 91	0 73	0 39

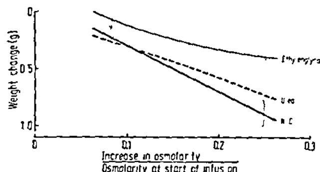


Fig 2 *Dose/response curves for 3 test substances* Weight reductions of the lungs reached after 45 sec of infusion are plotted against the induced osmolarity gradients, defined as the increase in perfusate osmolarity during infusion divided by the perfusate osmolarity at the start of that same infusion The osmolarity of the perfusate before any additions was always 290 mosm/l The vertical bars indicate the range of the observations

infusion divided by the perfusate osmolarity at the start of that same infusion. The weight reductions increased with the size of the osmolarity gradient induced. The responses to ethyleneglycol infusions were much smaller than those to urea and sodium chloride when related to the osmolarity gradients There was furthermore a tendency towards greater responses to sodium chloride than to urea infusions

A critical question to answer is the extent to which the weight changes observed represented reductions in vascular capacitance Loss of lung tissue water due to transcapillary flux of fluid in response to increased intravascular osmolarity could also be expected to cause weight reductions of the preparations In order to answer this question we infused the test solution of sodium chloride until a maximal weight reduction was obtained The one experiment of this type giving the largest weight response is depicted in Fig 3 and 4 The first of these two figures demonstrates the actual polygraph tracing, the second is a drawn out graph of the down slope from the weight recording To simplify our calculations we will assume that the lung tissue behaved as a perfect osmometer and that the pulmonary vessels were permeable to water only The wet weight of the lung preparation without any perfusate is about 7 g (Lunde 1967) and the percentage of water in bloodless rabbit lungs is certainly not more than 70 (Mountcastle 1968) Consequently 5 g of extravascular water must be a maximal figure for this preparation

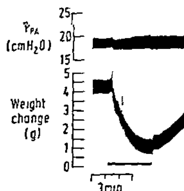


Fig 3 *Effects of a cumulative rise of plasma osmolarity* Isolated perfused rabbit lungs Flow 250 ml/min test solution sodium chloride 7000 mosm/l infusion rate 4 7 ml/45 sec Infusion period is marked by horizontal bar The two vertical dotted lines indicate the first 45 sec of the response time

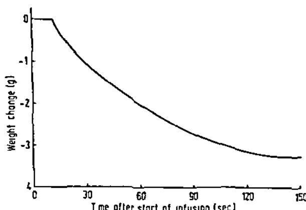


Fig 4 The figure shows a drawn out graph of the down slope of the weight recording demonstrated in Fig 3

During the first 45 sec of infusion, i e before any recirculation took place, 187 5 ml of plasma passed through the pulmonary vascular bed With the infusion-pump setting selected for this particular experiment 4 7 ml of a sodium chloride solution of 7000 mosm/l were infused during that same period The increment in milliosmoles must then have been 32 9 mosm in a total volume of 192 2 ml (187 5+4 7), that is an increment of 171 mosm/l Because of previous additions of ethyleneglycol plasma osmolarity before the start of perfusion was found to be 500 mosm/l In spite of this the lung preparation had a normal weight, indicating that no pulmonary edema was present Assuming that water only crosses the vessel wall we will have the following relationship

$$\frac{500}{500+171} = \frac{x}{5}$$

x is the extravascular water volume after a new osmotic equilibrium has been reached In this experiment x was 3 7, consequently the maximal transvascular waterflux was 1 3 ml during the first 45 sec of infusion If we turn to Fig 4 we will see that at this point the weight reduction of the preparation was 1 6 g and, furthermore, the fall in weight was still rapidly proceeding despite the fact that recirculation had not yet started

Since the vascular walls are not permeable to water only the osmotic gradient certainly must have been smaller than assumed in our calculations Furthermore, it is unlikely that the total lung water content is available for osmotic equilibration within such a short period of time These considerations will further tend to reduce the importance of transvascular water flux in causing the weight reductions observed It should also be stressed that if the total weight reduction obtained was due solely to dehydration of the lungs more than half of the total lung water content would have been lost We consider this to be highly improbable Thus, at least a part of the weight reduction must have been caused by a fall in intravascular volume

Discussion

The present experiments confirm the previous observations from cat that small and moderate elevations of plasma osmolarity are able to reduce pulmonary vascular resistance This effect appears to be mediated by way of a reduction in vascular smooth muscle tone (Hauge and Bø 1971) In the present preparation this response was in most cases a relatively long lasting one and would therefore, as a rule, only be obtained at the first infusion of a hyperosmolar solution In contrast, the effect of plasma hyperosmolarity of lung weight was present throughout the experiment, thus making it reasonable to conclude that the weight reductions observed were in dependent of changes in pulmonary vascular resistance It is, furthermore, unlikely that the weight responses were secondary to airway changes since bronchomotor reactions were not observed Although a part of the weight reductions observed might be due to dehydration of the lung, we believe that the experiment illustrated in Fig 3 and 4 proves that this cannot be the sole explanation of our finding Some reduction of pulmonary vascular capacitance must have occurred as a response to plasma hyperosmolarity

When the 3 test-substances are compared with regard to their ability to reduce the weight of the preparation, it is evident from Fig 2 that ethyleneglycol is the least effective one and that sodium chloride is the most potent one of the three Urea takes an intermediate position not much different from sodium chloride It is known that ethyleneglycol has the highest rate of penetration into smooth muscle cells of the 3 substances tested (Johansson 1969), and also that urea comes as no 2 in this respect The capillary permeability to sodium chloride and urea is, however about equal (Pappenheimer 1953) The differences observed may therefore reflect differences in e ability of the test substances to change the smooth muscle tone of capacitance vessels in the lung By increasing extracellular osmolarity in a rat portal vein Johansson and Jonsson (1968) showed that a fall in the smoth muscle cell volume was accompanied by inhibition of its electrical and mechanical activity Their observation is compatible with our finding that hyperosmolarity reduces PVR but it fails to explain the reduction in intravascular volume In this connection it is of interest to note that another stimulus, namely an elevation of plasma catecholamine levels, elicits the same response pattern in the pulmonary vascular bed as did infusion of hyperosmolar solutions in the present study (Hauge, Lunde and Waaler 1967) Both stimuli may be operative under various forms of circulatory stress and contribute to mobilization of blood from the lungs

The lung vasculature is known to react differently from systemic vascular beds in the response to various stimuli *e g* hypoxia, ATP and acidosis (Waaler Hauge and Lunde 1966) It may therefore be of interest to compare the effects of hyperosmolar solutions on capacitance vessels in the lung with the effect of this stimulus on the same functional type of vessels in muscle Although hyperosmolarity is a potent vasodilator stimulus in skeletal muscle (Mellander *et al* 1967), no effect on capacitance vessels in skeletal muscles has been detected (Mellander *et al* 1967) Apparently there is large segmental differences in the two vascular beds in the responses to a rise in intravascular osmolarity

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Acta physiol. scand. 1971. 82. 382—392

From the Department of Zoophysiology, University of Göteborg, Sweden

The Effects of Changes in the Ionic Environment on Venous Smooth Muscle Distribution of Sodium and Potassium

By

Bo Wahlstrom

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Abstract

Wahlstrom, B *The effects of changes in the ionic environment on venous smooth muscle distribution of sodium and potassium* Acta physiol scand 1971 82 382—392

The sodium potassium calcium and magnesium content in the portal vein of the rat was determined by atomic absorption spectrophotometry in different environmental conditions It was found that minute changes in the ionic environment caused considerable variations in the [illegible]a$^+$ K$^+$ and Ca^{2+} content and Na/K ratio Phosphate in the solution increased the sodium and calcium content In sodiumfree solution all muscle sodium was rapidly lost No bound sodium was detected The ionic content of portal vein was also dependent on dissection and blotting technique A standard procedure and a standard solution was therefore chosen In this solution the following values were obtained Na$^+$ 88, K$^+$ 52 Ca^{2+} 5 7 and Mg^{2+} 3 7 mmole/kg freshweight respectively
The ex [illegible] weight The calcu lated i [illegible] K$^+$ were 62 and 157 m[illegible] [illegible] that about 20 % of the [illegible] ^{42}K$^+$ showed that for the linear exchange of muscle potassium the rate constant was 0 85—0 90/hr
K low solution decreased the efflux of ^{42}K$^+$ in spite of increased electrical activity This suggests that the permeability to potassium is lowered Noradrenaline had no effect on the ^{42}K efflux in normal solution

Information about the distribution and movements of ions in vascular smooth muscle will provide a basis for a better understanding of the relationship between these and the spontaneous activity exhibited by the muscle in different environmental conditions The electrophysiology of the rat portal vein has been studied with the sucrose-gap technique (Axelsson *et al* 1966, 1967 a, 1970 in press) and successful impalements with microelectrodes have also been reported from a similar preparation (Nakajima and Horn 1967) The present work aimed at obtained information about the effects of changes in the external ionic environment and of drugs on the ionic

content and on $^{42}K^+$ movements This paper describes measurements of Na^+, K^+, Mg^{2+} and Ca^{2+} content, wetwt/freshwt, extracellular space and uptake and efflux of $^{42}K^+$ in the portal vein of the rat Some of the results have been presented to the XIII th Scandinavian Congress of Physiology in Göteborg, August 1969 (Wahlstrom 1969).

Methods

White rats, weighing 150—200 g were killed by a blow over the neck and bled out The abdomen was opened and 20—25 mm long pieces of the portal vein carefuly dissected out and freed from fat and peritoneum The vein was cannulated at the mesenteric end and 0 5 ml of calcium free Krebs solution (composition, see Table I) slowly injected Thereafter the vein was cut out from the animal, gently squeezed and the freshweight determined on a torsion

paper and apply a 2 g weight for 3 sec

Determination of ionic content

The tissue was placed in an acid washed borosilicate test tube and digested in 0 25 ml of TCA GAA, 1 g/ml, or 2 ml of H_2O_2 NH_4OH, 4 1 Each sample was then diluted to a final volume of 10 ml with distilled H_2O and the Na^+, K^+, Ca^{2+} and Mg^{2+} measured in an atomic absorption spectrophotometer (Techtron) In some experiments a flame photometer (Eppendorf) was used

Uptake experiments

Muscles were
$^{42}K^+$ activity c
sample was di
the ^{14}C activi
used for determination of ionic content

Efflux experiments

Muscles incubated for 3 hrs in a Krebs solution containing $^{42}K^+$ were passed through series of test tubes containing inactive oxygenated Krebs solution Test solutions could be inserted at

TABLE I Composition of Krebs solution, obtained by mixing isotonic (310 mosmoles/l) stock solutions Values are expressed as ml per 1000 ml of Krebs solution

Isotonic solutions	A	B	C	D	E	F	G
KCl, ml	38 3	38 3	38 3	38 3	—	—	38 3
LiCl, ml	—	—	—	—	—	—	850 5
$CaCl_2$, ml	24 2	24 2	24 2	—	24 2	24 2	24 2
$MgCl_2$, ml	11 6	11 6	11 6	11 6	11 6	11 6	11 6
$NaHCO_3$, ml	100 0	38 3	100 0	100 0	100 0	100 0	38 3
Na_2HPO_4, ml	7 7	—	—	—	—	—	—
Glucose, ml	37 1	37 1	37 1	27 1	37 1	37 1	37 1
NaCl, ml	781 1	850 5	788 8	818 0	788 8	788 8	—
^{42}KCl, ml	—	—	—	—	38 3	38 3	—
^{14}C-sorbitol, ml	—	—	—	—	3 2	—	—
O_2/CO_2, %	97/3	99 1	97/3	97 3	97/3	97/3	99 1

The pH in all solutions was kept at 7 4

various points during the washout series *The activity in each tube was counted* in a scintilla tion counter (Beckman) In some experiments the radioactivity in the tissue at the end of the efflux was determined This activity could be added to the activity of successive samples in reverse [illegible] th values [illegible] in estimat [illegible]

The [illegible] isotopes were supplied by the Radiochemical Centre Amersham L-nor adrenaline was obtained as the bitartrate (Merck)

Results

Ionic content in Krebs solution

The first step towards defining optimal conditions was to compare different tech niques of determining wetweight and freshweight to each other and to study the effect of different salt solutions on the ionic content Table II summarizes the results from an experiment in which three different solutions two different blotting tech niques and two different dissection procedures have been compared to each other The incubation period was the same for all muscles, namely 3 hrs The composition of each solution and the weighing techniques are described in Methods As can be seen from Table II incorporation of phosphate in the solution (group 1) increased the Ca° content Results from other experiments with this solution showed that the Ca° content of the tissue increases with time when phosphate is present

A comparison between group 1, 2 and 3 reveals a considerable variation in ionic content of the portal vein in different physiological salt solutions Group 2 had the highest Na content significantly different from group 3 ($p < 0\ 05$) It was also nd that there were considerable variations in the pH of this solution The total buffer capacity is only one third of that of the other solutions and small in homogenities in the gas composition and/or the gas distribution in the organ bath may give rise to unwanted fluctuations in the pH, that do not occur in the other solutions Solution A and B were for the abovementioned reasons not used in the experiments described below

TABLE II The ion contents in mmoles/kg freshwt the wetwt/freshwt ratio and the Na/K ratio of rat portal veins given as mean values with S E of the mean The composition of the var ous solutions is given in Methods

Group Solution	1 A	2 B	3 C	4 C	5 C
O_2/CO_2 %	97/3	99/1	97/3	97/3	97/3
Na^+, mmole/kg W_{Fr}	70 6 ±3 1	87 2 ±6 7	68 3 ±2 3	57 7 ±1 3	69 8 ±1 4
K^+,	33 6 ±1 6	35 2 ±1 6	31 5 ±1 4	33 5 ±1 3	28 2 ±2 6
Ca^{2+}, ,	7 0 ± 7	5 4 ± 5	4 9 ± 7	4 6 ± 6	5 1 ± 5
Mg^{2+}, ,	3 6 ± 3	3 5 ± 1	3 5 ± 1	3 4 ± 1	3 3 ± 1
W_W/W_{Fr}	79 + 03	84 ± 03	87 ± 03	80 ± 02	75 ± 01
Na/K	2 11 ± 12	2 51 ± 26	2 18 + 16	1 73 ± 05	2 65 ± 29

n = 6
C Veins in this group were blotted with filter paper
C Veins in this group were slit open along the whole length of [illegible]ve

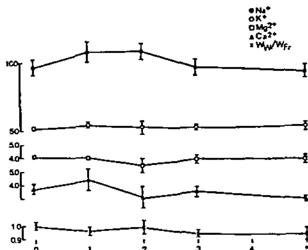


Fig 1 The ionic content of the rat portal vein after different exposure times to Krebs solution Abscissa time in hours Ordinate the four upper curves ion content in mmoles/kg freshwt the lower curve wetwt/freshwt ratio The vertical bars represent SE of the mean n = 6

A comparison between group 3, 4 and 5 shows that veins in group 4 had a significantly lower Na^+ content and Na/K ratio ($p < 0.02$) that the other 2 groups and also a lesser scattering of values around the mean as shown by the smaller standard deviations Solution C was therefore used in the following experiments with the filter paper blotting technique which can be accurately standardized by choosing an appropriate time and weight to be applied

Steady state experiments

A prime condition for our experiments was that the muscle must exhibit steady state behaviour, *i e* no net changes in ionic content should take place during the course of an experiment

Fig 1 shows the ion content of portal veins after different periods of incubation in solution C In this experiment the sodium content rose from 95 mmoles/kg freshwt immediately after dissection to 108 mmoles/kg freshwt after one hour in the solution After 3 hrs the sodium content was back to 95 mmoles/kg freshwt and was stable for another 3 hrs The potassium content remained relatively constant throughout the experiment about 50 mmoles/kg freshwt The Na/K ratio was 1 90 after 3 hrs The calcium content showed a slight increase to 4 4 mmoles/kg freshwt after 1 hr but fell again to 3 mmoles/kg freshwt after 5 hrs an exceptionally low value for this tissue The wetwt/freshwt ratio did not change significantly

The sensitivity of the portal vein to experimental treatment is shown in Fig 2 taken from a similar experiment in which the tissues were deliberately hurt during the dissection It can be seen from the figure that sodium content increases throughout the experiment up to about 120 mmoles/kg freshwt accompanied by a decrease in the potassium content from 45 to 25 mmoles/kg freshwt during the same period The Na/K ratio was thus 4 8 after 5 hrs The Ca^+ content increased significantly from 4 1 to 6 3 mmoles/kg freshwt

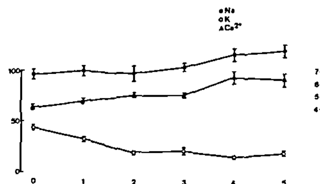


Fig 2 The effect of damaging the tissue on ionic content Abscissa time in hours Ordinate ionic contents in mmoles/kg freshwt Vertical bars represent SE of the mean n = 6

The effect of Na^+ free solution on the ionic contents

In sodium free lithium solution (composition see Methods) the sodium content decreased rapidly from 95 to 2 mmoles/kg freshwt in 5 min (see Fig 3) Within 1 min 75 mmoles, mainly extracellular sodium, left the muscle For the longer exposure times no definite values for the sodium content could be obtained Thus very little sodium was left in the tissue indicating that most of the sodium was extracellular The lithium content rose from zero to 115 mmoles/kg freshwt during the first 5 min followed by a slow rise up to 125 mmoles/kg freshwt during the next 25 min At the same time the potassium content fell by 9 mmoles/kg freshwt

K^+ uptake and extracellular space

The uptake of $^{42}K^+$ and ^{14}C sorbitol in portal veins was studied for periods up to 5 hrs The sorbitol uptake showed an initial rapid phase due to rapid diffusion of the tracer into the extracellular space which was completed in a few min After 10 min changes in the sorbitol space were small and insignificant We have therefore used the 10 min sorbitol space for calculations of intracellular ion concentrations as representing approximately the ECS_0

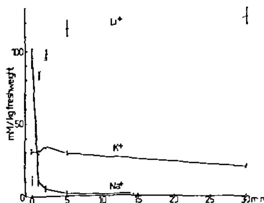


Fig 3 The effect of sodium free lithium solution on the ionic content Abscissa incubation time in minutes Ordinate ion content in mmoles/kg freshwt Vertical bars represent SE of the mean n = 5

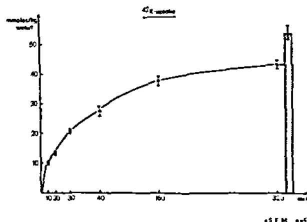


Fig 4 The uptake of $^{42}K^+$ in the rat portal vein Abscissa time in minutes Ordinate specific activity in mmoles/kg freshwt The vertical bars represent SD The shaded column shows the total K^+ content determined by atomic absorption spectrophotometry Each point is the mean of 5 muscles

The uptake of $^{42}K^+$ showed a similar rapid initial phase (see Fig 4) After 10 min the potassium space was about 1300 ml/kg freshwt About half of the exchangeable potassium was taken up in 40 min after correction had been made for tracer K^+ in the extracellular space The relative activity, expressed as the percentage ratio between the potassium content calculated from the $^{42}K^+$ counts and the potassium content determined by atomic absorption spectrophotometry increased to a maximum value of 81 % which was obtained after 5 hrs incubation

The Na^+, K^+, Ca^{2+} and Mg^{2+} contents of the muscle were determined in the same experiment The results are summarized in Table III

From the ionic contents and the ECS_{10} the intracellular concentrations of sodium and potassium were calculated The results are summarized in Table IV together with the equilibrium potentials calculated from the Nernst equation The equilibrium potential for potassium is considerably more negative than the actual membrane potential measured with intracellular microelectrodes for which values between −41 and −62 mV have been reported (Nakajima and Born 1967) but agrees closely with values calculated by Casteels (1969) on the taenia coli Calculation of extracellular sodium and potassium showed that of the total sodium content 90 % is extracellular while only 8 % of the total potassium is dissolved in the extracellular space

TABLE III Total ion content (mmoles/kg freshwt and wetwt/freshwt ratio of portal veins, given as mean values with S E of mean. The number of observations is given in brackets

Na^+ mmole/kg freshwt	87 9 ±4 6 (34)
K^+ mmole/kg freshwt	52 1 ±3 4 (35)
Ca^{++} mmole/kg freshwt	5 7 ± 4 (33)
Mg^{++} mmole/kg freshwt	3 7 ± 1 (35)
Wetwt/freshwt	85 ± 06 (35)

TABLE IV Intracellular ion concentrations in mmoles per litre intracellular fluid (mM) and equilibrium potentials in mV

ECS_{10}, ml/kg freshwt	490
$[Na^+]_i$, mM	62
$[K^+]_i$, mM	157
E_{Na^+}, mV	+21
E_{K^+}, mV	−86

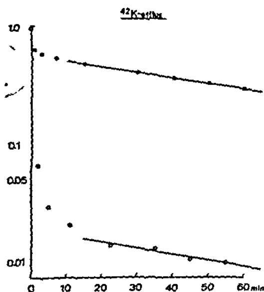
The dry substance was estimated to 200 ml/kg freshwt

Efflux experiments

a) $^{42}K^+$ efflux in normal solution

The efflux of tracer potassium was studied by incubating portal veins in ^{42}K solution for 3 hrs (composition see *Methods*) and then following the washout of tracer in nonactive solution The effluent and muscle counts were plotted against time on semi logarithmic paper (Fig 5)

The washout curve consisted of an initial rapid phase which can be attributed to extracellular tracer transferred superficially with the muscle and a slow linear phase The rate constants for the linear phase was 0 90/hr for the effluent counts and 0 85/hr for the muscle counts The slopes of the two curves did not differ significantly during this phase and the efflux thus followed a single exponential The values



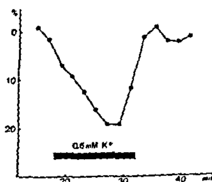


Fig 5 Fig 6

Fig 5 The efflux of ^{42}K from the rat portal vein in Krebs solution Abscissa time after [illegible] between [illegible] ed

Fig 6 The effect of K low solution on the efflux of $^{42}K^+$ Abscissa time in min Ordinate percentage difference between effluent counts in normal and in K^+ low solution Horizontal bar shows the incubation period in K low solution Each point is the mean of 5 muscles

from 6 different muscles were used to estimate the size of the linearly exchanging fraction. We found that this fraction constituted about 50 % of the total muscle potassium.

b) $^{42}K^+$ efflux in K^+-low solution

Earlier experiments had shown that a decrease in or a removal of the external potassium caused a transient excitation usually accompanied by depolarization (Axelsson *et al.* 1967 a). The effect of K^+-low solution on the $^{42}K^+$ efflux was therefore studied with this observation borne in mind. Six muscles were loaded with $^{42}K^+$ for 3 hrs and the washout of tracer followed for 45 min. The muscles were then reloaded for 1 hr and the effect of the tracer studied for another 45 min. A 15 min period of K^+ low solution was inserted during the linear part of the second washout. Control and test curves from the same muscle could then be compared to each other. As can be seen in Fig. 6 the decrease in the external potassium concentration caused a decrease in the efflux of potassium. The fall in the rate of efflux was not instantaneous reaching a maximum of 20 % after 7—8 min and a partial return to normal values can be seen towards the end of the test period. After return to normal solution the efflux also returned to normal.

c) The effect of noradrenaline on the efflux of $^{42}K^+$

Muscles were loaded in tracer solution for 1 hr and the washout of tracer in non-active solution followed for 30 min. This procedure was repeated twice, and from each muscle we thus obtained three curves, one control and two noradrenaline curves. A solution containing noradrenaline, 10^{-7} g/ml, was inserted for 5 min during two parts of the efflux curve, a) in the rapid initial phase, and b) in the later linear phase.

Noradrenaline had no significant effect on the efflux of $^{42}K^+$, neither during the initial phase, nor during the linear phase of the washout curve.

Discussion

During the last few years information about the ionic content and distribution in various smooth muscle types has accumulated (Bülbring *et al.* 1963, Casteels and Kuriyama 1966, Rorive 1967, Washizu 1969). There is general agreement on some points which will be briefly summarized here. First of all the total muscle content of Na, expressed as mmoles per kg freshwt, netwt of drywt is higher than in skeletal muscle and nerve. The K content is lower in some cases as low as 20—25 mmoles/kg wetwt (Rorive 1967). These differences between skeletal muscle and smooth muscle have been attributed to the large extracellular space usually found in the latter type of tissues (Goodford 1964, Bülbring *et al.* 1968, Konold *et al.* 1969, Jones and Karreman 1969) as calculations of intracellular ion concentration based on simultaneous measurements of sodium and potassium content and extracellular space determinations give values of (Na) and (K) close to or equal to those reported from skeletal

muscle The E_K is also considerably more negative than the actual membrane potential (Casteels and Kuriyama 1966, Kao and Nishiyama 1969 Bulbring *et al* 1968)

All the abovementioned characteristics of smooth muscle i e high Na^+ content low K^+ content and large extracellular space has been found to apply to the rat portal vein as can be seen from Table III and IV

Nakajima and Horn (1967) measured the resting and action potential of the superior mesenteric vein of the rat with intracellular microelectrodes Their preparation differs only slightly from our They recorded action potentials with an amplitude ranging from 35 to 59 mV, with occasional overshoot The resting potential i e the maximum polarization between bursts was −51 mV The value for the transmembrane potassium potential calculated in Table IV is −86 mV thus considerably greater than the measured potential This difference can be explained if the sodium permeability is relatively high as indicated by the extremely rapid loss of sodium in sodium free solution An alternative explanation is a low potassium permeability The relatively slow exchange of potassium indicates that this possibility must not be overlooked

The sodium potassium ratio in vascular smooth muscle is altered by the dissection trauma (Rorive 1967, Konold *et al* 1969) This is the case also for the portal vein as clearly shown by a comparison of Fig 1 and 2 above The ionic content is also sensitive to small changes in the ionic composition of the extracellular milieu as shown in Table II These findings implicate that careful evaluation of technique and solution is needed when values for ionic content from different authors are compared o each other Our criteria for a good solution were a low Na/K ratio and a low ^{2} content This choice was not arbitrary since it is known from intestinal smooth uscle that damaged muscles accumulate sodium and calcium (Goodford personal comm)

The sodium distribution in the rat portal vein seems to be quite simple i e a large extracellular amount due to high $(Na)_o$ and a large extracellular space a rapidly exchanging fraction and very little if any bound sodium Buck and Goodford (1966) in a similar experiment on strips of guinea pig taenia coli estimated the fraction of bound sodium to be 6 mmoles/kg freshwt These authors also found that lithium accumulated in the cell in sodium free lithium solution while potassium was lost This is also the case for the rat portal vein and the almost quantitative accordance between lithium gained and potassium lost seems to lend support to the suggestion put forward by Keynes and Swan (1959) that the sodium pump mechanism in the membrane of the muscle whether striated or smooth discriminates between lithium and sodium and in order to maintain isoosmolarity the cell forces potassium through the membrane

The potassium distribution in smooth muscle tissues has often been described by a two compartmental model the membrane acting as a simple diffusion barrier between an intracellular and an extracellular solution Recently proposals for a new model have been made (Jones and Karreman 1969 b) and various authors have also tried to explain deviations from the two-compartmental model by including one

or more extra compartments, arriving at double or triple exponential equations for in and efflux From the shape of the curve shown in Fig 4 it seems likely that there is a large fraction of potassium in the portal vein, around 20 % of total potassium content, that does not exchange with $^{42}K^+$ or does so at an extremely slow rate This fraction is not necessary intracellular but may be bound to the network of extracellular collagen and elastin fibres abundant in this tissue

The potassium efflux as shown in Fig 5 however is easily explained by the two compartmental model, with a large fraction, roughly 50 % of total muscle potassium, exchanging linearly Similar values have been obtained from the taenia coli of the guinea pig (Goodford and Hermansen 1961) Since the washout time was only 1 hr the slow fraction was not likely to interfere with the calculations

Axelsson *et al* (1967) reported that decrease in the external potassium concentration to less than 2 mM increased the frequency of the spontaneously occurring bursts in the rat portal vein. Similar observations were made in taenia coli of the guinea pig (Axelsson and Holmberg 1970, in press) These findings seemed to refute the generally accepted hypothesis about the relationship between log $(K^+)_0$ and E_m The results reported above, however, shows that there is a decreased efflux of $^{42}K^+$ in K^+ low solution, indicating a decreased membrane permeability towards this ion This effect counteracts the influence of the increased potassium gradient on the membrane potential For the following reasons we believe that the decrease in potassium permeability is in fact greater than shown in Fig 6 namely that a) the depolarization in itself, all other parameters being equal, would tend to increase the K^+ efflux because of the lesser electrostatic attraction of the membrane on the escaping K^+ ions and b) the increased electrical activity itself would also lead to a greater efflux of K^+ ions

Our trials to detect any change in the efflux of $^{42}K^+$ on application of noradrenaline gave only negative results Increased potassium efflux caused by noradrenaline has been reported in depolarized taenia coli (Jenkinson and Morton 1967) and in normally polarized taenia coli (Bulbring and Tomita 1969) It is possible that variations in the spontaneous activity in this case mask permeability changes Another explanation might be that potassium movements are unaffected by noradrenaline in this preparation but that other ions should be considered

Part of the work was made at Dr P Goodford s laboratory at the Wellcome Foundation Research Laboratories Beckenham Dr Goodford s generous help is grateful acknowledged

My thanks are due to Mrs Gunilla [illegible] for excellent technical assistance

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From the Institute of Physiology, University of Oslo Norway

Pulmonary Transcapillary Exchange of ^{24}Na and ^{51}CrEDTA. An Evaluation of Factors Influencing the Extraction of these Tracers during one Passage through an Isolated Lung Preparation

By

GUNNAR NICOLAYSEN

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Abstract

NICOLAYSEN, G *Pulmonary transcapillary exchange of ^{24}Na and ^{51}CrEDTA An evaluation of factors influencing the extraction of these tracers during one passage through an isolated lung preparation* Acta physiol scand 1971 *82* 393—405

Experiments with the single injection indicator diffusion method have been carried out in an isolated, ventilated and plasma perfused rabbit lung preparation. A mixture of two diffusible tracers (^{24}Na, ^{51}CrEDTA) and one intravascular reference tracer (^{125}I albumin) were used [illegible] venous outflow fractions collected in [illegible] he diffusible indicators A small extra [illegible] e most important determinator for the [illegible] age through the vascular bed Results from several types of experiments were consistent with there being also some moderate degree of diffusion limitation for the extraction of the diffusible tracers Extraction of the diffusible tracers appeared to be moderately increased when the intravascular concentrations of both Ca^{++} and Mg^{+} were reduced to such low levels that edema was developing in the preparation It appears unlikely though that the single injection indicator diffusion method in its present form can yield more detailed information on capillary permeability to small solutes in this rabbit lung preparation

Marked simultaneous reductions in intravascular concentrations of ionized calcium and magnesium lead to edema formation in an isolated lung preparation (Nicolaysen 1971 a) A more moderate reduction in the concentration of ionized calcium in the perfusate has been shown to increase the hydraulic conductivity of the capillaries in the same preparation (Nicolaysen 1971 b) One explanation for these two sets of findings could be that the permeability of the endothelial cells and of the intercellular clefts of the endothelium are differently affected by reductions in perfusate concentrations of ionized magnesium and/or ionized calcium (Nicolaysen 1971 b) Investigations on the capillary permeability to small solute molecules under

conditions of reduced concentrations of perfusate calcium and magnesium could possibly reveal further information on this point

Several investigators have studied the transcapillary exchange of small solute molecules and ions in the pulmonary circulation with the single injection indicator diffusion technique (Chinard and Enns 1954, Bauman *et al* 1957, Rothschild *et al* 1959, Chinard, Enns and Nolan 1962) Chinard *et al* (1954) and Bauman *et al* (1957) found that only small amounts of ^{22}Na passed out from the pulmonary vessels of dogs and healthy humans, respectively, during one single passage of the tracer through the pulmonary vascular bed Such a small extraction from the capillaries during the early phase of one single passage could be due to a small extravascular space of distribution to an insufficient contact time and/or to low capillary permeability to the test substance Both groups of investigators reached the conclusion that *the extravascular space of distribution had not been the limiting factor* for the small exchange observed in their studies

On the basis of these previous investigations the single injection indicator diffusion method seemed suitable for a study of possible changes in lung capillary permeability upon alterations in perfusate concentrations of ionized magnesium and/or calcium However preliminary experiments indicated that the extravascular space of distribution did limit the amount of small solute tracer molecules diffusing out of the exchange vessels during one single passage through the isolated rabbit lung preparation used

Some investigations on capillary permeability at very low levels of ionized calcium and magnesium were carried out However the larger part of the present investigation was turned into a more methodological study The results obtained indicate that the extravascular space of distribution was the most important determinator for the degree of transcapillary exchange of ^{24}Na and $^{51}CrEDTA$ during only one single passage through this isolated rabbit lung The permeability characteristics of the lung capillaries appeared to influence such transcapillary exchange to a moderate extent only

Methods

Rabbits weighing 2 5—3 5 kg were anesthetized with i v injections of pentobarbitone (Nembutal® (30—50 mg/kg)) and in addition given heparin (*750 mg/kg of dissolved pure powered heparin (Novo)*) *intravenously During a period of positive pressure ventilation* (through a tracheostomy cannula) the heart and lungs were dissected free and removed from the animal The left auricle and the pulmonary artery were cannulated (Nicolaysen 1971 a)

10—14 min after the rabbit s own circulation had been stopped the perfusion of both lungs was started At the same time pos tive pressure ventilation was also initiated using peak inspiratory and end expiratory pressures of 10 and 1—2 cm of water respectively The ventilation gas was a mixture of 5 % CO_2 in air The perfusate flow through the preparation was adjusted to about 270 ml/min from the outset of all experiments The pulmonary arterial pressure was then always below 20 cm of saline The flow was kept constant thro ghout each experiment except in one group of preparations where the flow was halved for certain periods A Harvard peristaltic pump (Model 1210) was used for the perfusion the two roller slits being used in parallel whereby a nearly non pulsatile flow was obtained

The pulmonary arterial pressure (P A p) was continuously recorded with a Statham pressure transducer P23Db *The weight changes of the preparation* were continuously followed via a Sanborn force transducer FTA 100-1 underneath which the preparation was suspended (N co-

laysen 1971 a) In order to avoid vasomotor reactions 7.5 mg papaverine sulfate was added to the perfusate a few min after start of perfusion The left atrial pressure (L.A p) was in some experiments purposely altered for shorter intermediate periods, but was otherwise kept constant at 0 5—1 mm of Hg

[illegible] perfusate (Nicolaysen 1971 a) This [illegible]) I U./ml of pure powdered heparin [illegible] 1000 × *g* The plasma obtained was [illegible] rt of an experiment a plasma batch [illegible]

[illegible] The following test substances (tracers) [illegible]

activities in these same samples were measured 7—8 days later The samples were kept frozen in this time interval There was no admixture in the ^{24}Na-channel from ^{125}I or ^{51}Cr at the gain and discriminator settings used The ^{24}Na activities in the samples were negligible when the ^{125}I and ^{51}Cr activities were measured The admixture from ^{51}Cr in the ^{125}I channel was corrected for The ^{125}I gave no admixture in the ^{51}Cr channel The activity of each isotope was corrected for decay

[illegible]

the 24 countings thus performed

In each fraction of the venous outflow *the activity of each isotope* (C_s) *was normalized* with respect to the activity of that same isotope in the injectate This was done by dividing the count rate in the sample by the count rate in the undiluted injectate (C_i)

A fractional extraction was determined for the diffusible tracers (^{24}Na, ^{51}CrEDTA) in each of the outflow fractions The fractional extraction (Crone 1963) is defined as $(C_r—C_d)/C_r$, where C_r and C_d are the normalized concentrations (C_s/C_i) in the same outflow fraction of reference and of diffusible tracer respectively

Evaluations of extracted amounts of diffusible tracers were also carried out After a bolus [illegible] there will at [illegible] concentration [illegible] acers from the [illegible] to evaluate, in [illegible] lar bed in the early phase of each tracer injection test before net backdiffusion started These relative estimates are in the following termed the "*evaluated extracted amount*" The evaluations were made by making a plot of $C_r—C_d$ in each outflow fraction against the fraction number A curve was drawn between these points at the best visual fit, and the curve extrapolated back to the zero level of $C_r—C_d$ (Fig 2) The area between this curve and the zero level of $C_r—C_d$ was next cut out and the piece of paper weighed The extent of these areas must be proportional to the true extracted amounts as long as the volume in the outflow fractions were equal The "evaluated extracted amount" was therefore used for comparisons between different tests in the same experiment

In some experiments it was attempted to vary the size of the extravascular space of the preparation during the perfusion period After a period where L A p had been kept at 0 5—1 mm Hg this pressure was suddenly increased by 5 (1 exp) or 10 (2 expts) mm Hg This was achieved by diverting the outflow up a vertical glass ladder of desired length When about 55 sec had elapsed, a tracer injection test was carried out As soon as the collection of the outflow fractions had been completed, L A p was reduced to its initial low value About 40 min later the L A p was again increased in the same way and to the same level At such increases in L A p a marked weight increase of the preparation takes place At first this increase is rapid It gradually becomes less marked and after about 4 min the increase in weight is about linear The first part of this weight increase mostly reflects net capacity changes of the vessels in the preparation whereas the later, linear part of the increase reflects net capillary filtration (Nicolaysen 1971 b) The rate of weight increase at this late stage was assumed to give the rate at which the extravascular fluid volume increased during the whole period of elevated L A p Thus by watching this rate of rise and by controlling the length of the period of increased L A p one could obtain a defined increase in extravascular fluid volume When this parameter had increased by the desired amount a second tracer injection test was performed Next the L A p was again reduced to its initial low value A third test exactly similar to the first one was applied about 40 min later All the three tracer injection tests were thus carried out under the same flow and pressure-conditions the extravascular fluid volume being increased by a known amount during the second one

Increased contact time between tracers and exchange vessels In three experiments it was attempted to study the effects of changes in the contact time between diffusible tracers and the walls of the exchange vessels The experiments started as usual with a perfusate flow of 270 ml/min A first tracer injection test was performed after about 20 min of perfusion There after the flow through the preparation was reduced to about one half of its previous value A period of 20—40 min was allowed for adjustments in vascular pressure and a second tracer injection test was then performed The flow was next increased to 270 ml/min and a third test carried out after a period of 20—40 min with this flow The rate of fraction collection was 3 samples per sec during the first and the third test 1 5 samples per sec during the second

Induced edema formation and its reversal Edema develops at physiological intravascular pressures in the present preparation when enough EDTA is added to the perfusate for the concentration of EDTA to exceed the sum of the concentrations of total (ionized plus non ionized calcium) (Ca_t) and total magnesium (Mg_t) by about 20 % (Nicolaysen 1971 a) In its earlier stages this edema development is reversible (Nicolaysen 1971 a) In three experiments such edema development was induced and thereafter reversed The experiments were in the usual way and ordinary perfusion carried out for about 20 min In the later part of this period one tracer injection test was performed A solution of EDTA in saline 67 mM) was then added to the plasma perfusate so as to give a concentration of EDTA of about 4 5 mM (≈130 % of ($[Ca_t] + [Mg_t]$)) About 40 min after this EDTA addition and at a time when the preparation showed an accelerating weight increase the second tracer injection test was carried out At this point the preparations had gained about 15 g in weight Immediately after completion of the fractionated outflow collection of the second tracer injection test the edema development was stopped by addition of a $CaCl_2$ solution (0 1 M) to the perfusate This addition of $CaCl_2$ also tended to cause some increase in P A p This effect was eliminated by injection of 10—25 μg of adrenaline into the pulmonary arterial tubing The vascular pressures were thereby kept almost unchanged Subsequent to the $CaCl_2$-addition and the reversal of the edema formation a third tracer injection test was carried out in the usual way

Results

Three tests with bolus injections of the isotope mixture and subsequent fractioned outflow collections were carried out in each of two control preparations In these experiments flow L A p and perfusate composition were kept unchanged throughout the experiments The individual tests in each experiment were carried out at about 40 min intervals A slow and gradual decline in weight of the preparations was observed during the whole of the perfusion period

Fig 1 (lower part shows the normalized concentrations (see Methods) of the 3 tracer substances in the successive outflow fractions from one of the tracer in-

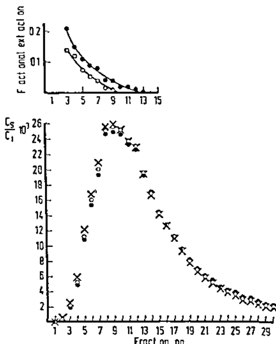


Fig 1 Lower part *Normalized tracer concentrations (C_s/C_i) in the outflow fractions after single injection of an isotope mixture (injectate) into the arterial stream of an isolated rabbit lung preparation* C_s = counts per min in outflow fraction C_i = counts per min in injectate Intravascular reference tracer × ^{125}I albumin Diffusible tracers ○ ^{51}CrEDTA ● ^{24}Na

Upper part *Fractional extractions of ^{24}Na and of ^{51}CrEDTA in those outflow fractions with positive values for this parameter* The fractional extraction is defined as $(C_r$-$C_d)/C_r$ where C_r and C_d are the normalized concentrations of the reference tracer and diffusible tracer *respectively* Illustration made from results in one of the tests in a control experiment

jections in a control experiment It will be seen that in the earlier outflow fractions the normalized concentrations of the diffusible tracers ^{24}Na and ^{51}CrEDTA were lower than those of the intravascular tracer ^{125}I albumin Furthermore, the normalized concentration of ^{24}Na was lower than that of ^{51}CrEDTA in these early fractions In the later fractions this pattern was reversed, with ^{125}I albumin showing the lowest values This general pattern of relationship between the normalized concentrations of the three tracers in the outflow fractions was the same in all the 6 tests performed in the 2 control preparations Such a picture of the smaller tracer molecules having the lowest normalized concentrations in the early fractions of the outflow, but the highest values in the later fractions is consistent with net outward transvascular diffusion taking place initially with a subsequent return of these smaller molecules to the vascular bed

In the upper part of Fig 1 is plotted the fractional extractions $((C_r$ $C_d)/C_r$ see Methods) for ^{24}Na and ^{51}CrEDTA in the early outflow fractions where the normalized concentrations of these tracers were lower than those of ^{125}I albumin All the tests in the control preparations showed the same pattern of fractional extraction for the diffusible tracers The rapid decline in fractional extraction for the two diffusible tracers should be noted It is furthermore seen that the fractional extraction for ^{51}CrEDTA became zero before that for ^{24}Na a general finding in these experiments This rapid decline in fractional extraction with rising concentrations of tracers (Fig 1) strongly suggests that the extravascular space of distribution is a

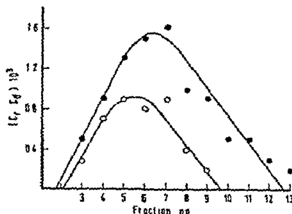


Fig 2 Graphs for evaluation of the extracted amounts of diffusible tracers in the tracer injection test described and illustrated in Fig 1 The individual points give 10^3 times extracted amounts (C_r-C_d) of ^{24}Na (●) and of $^{51}CrEDTA$ (○) in successive outflow fractions curves between each family of points fitted by eye The area between the curve for ^{24}Na and the abscissa was cut out and weighed to give what is called "evaluated extracted amount" for this tracer The same procedure was followed for $^{51}CrEDTA$ Abbreviations as in Fig 1

limiting factor for the amount of diffusible tracers passing out of the capillaries in the early part of the test

For the test depicted in Fig 1 the amount extracted for each of the diffusible tracers was evaluated as described in Methods Fig 2 shows the plot of the positive values for C_r-C_d in subsequent outflow fractions Similar plots were made for all the tests in the control experiments, and the areas thereby defined—the "evaluated extracted amounts"—were determined by weighing as described in Methods In Fig 3 is shown the "evaluated extracted amounts" thus found in all the tracer injection tests performed in the control experiments It will be seen that in each experiment the value for each tracer were well reproducible, with a slight rising tendency with time

Tracer injection tests were carried out also in three groups of subsequently performed experiments, with variations of extravascular fluid volume variations in perfusate flow and with induced edema formation In every injection test was the pattern for normalized concentrations of the tracers in the outflow fractions similar to that seen in the control experiments. The fractional extractions of the diffusible tracers did also show the same rapid decline as in the control experiments in all of these subsequent tests

The effect of changes in extravascular volume on the "evaluated extracted amounts" of diffusible tracers The control experiments indicated relative stability of the preparation as regards "evaluated extracted amounts" in successive tests (Fig 3) It was therefore attempted to study the effects of changes in extravascular volume on the "evaluated extracted amount" of diffusible tracers using each preparation as its own control In each of three preparations three tests with tracer injections and fractionated outflow collections were performed All these tests were performed under conditions of increased L A p, the first and the third tests shortly after the increase in L A p had been established The second tracer injection test in each experiment was carried out when the increased L A p had lasted for such a length of time that an increase in extravascular fluid volume of about 1 0—1 5 g had taken

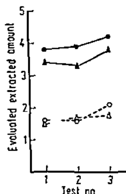


Fig 3

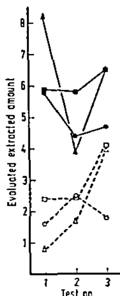


Fig 4

Fig 3 *"Evaluated extracted amounts" for ^{51}CrEDTA and ^{24}Na in three successive tracer injection tests in two control experiments* Isolated plasma perfused rabbit lung preparations The "evaluated extracted amounts" for the two diffusible tracers ^{51}CrEDTA and ^{24}Na were determined in each test as described in the text and in Fig 2 The "evaluated extracted amounts" are given in arbitrary units The three tracer injection tests were carried out at 40 min intervals

●——● Values for ^{24}Na in exp no 1
○——○ Values for ^{51}CrEDTA in exp no 1
▲- - -▲ Values for ^{24}Na in exp no 2
△- - -△ Values for ^{51}CrEDTA in exp no. 2

Fig 4 *"Evaluated extracted amounts" for ^{51}CrEDTA and ^{24}Na in three successive tracer injection tests in each of three experiments with temporary increase in extravascular fluid volume* Isolated plasma perfused rabbit lung preparations The "evaluated extracted amounts" for [illegible]

▲——▲ Values for ^{24}Na in exp no 3
△- - -△ Values for ^{51}CrEDTA in exp no 3
■——■ Values for ^{24}Na in exp no 4
□- - -□ Values for ^{51}CrEDTA in exp no 4
●——● Values for ^{24}Na in exp no 5
○- - -○ Values for ^{51}CrEDTA in exp no 5

place (see Methods) The net rate of capillary filtration at the moments of these second injections were 0 07, 0 2 and 0 2 g/min respectively The third elevation of L.A p with its third tracer injection test was not performed until the weight of the preparations had declined to the level prevailing before the second increase in L.A p

Fig 4 gives the "evaluated extracted amounts" of diffusible tracers in the tracer injection tests of these filtration experiments" In all but one case was the "evalu-

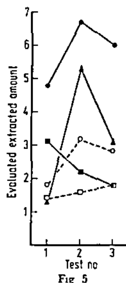


Fig 5

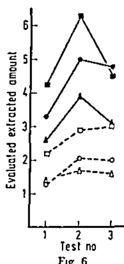


Fig 6

Fig 5 *"Evaluated extracted amounts" for ^{51}CrEDTA and ^{24}Na in three successive tracer injection tests in each of three experiments with flow variations* Isolated plasma perfused rabbit lung preparations The 'evaluated extracted amounts' for ^{51}CrEDTA and ^{24}Na were determined as described in the text and in Fig 2 and plotted in the same arbitrary units as in Fig 3 Tests no 1 and 3 in each experiment are control tests The second test was carried out under conditions of halved flow through the preparation (see text)

●——● Values for ^{24}Na in exp no 6
○- - -○ Values for ^{51}CrEDTA in exp no 6
■——■ Values for ^{24}Na in exp no 7
□- - -□ Values for ^{51}CrEDTA in exp no 7
▲——▲ Values for ^{24}Na in exp no 8

Fig 6 *"Evaluated extracted amounts" for ^{51}CrEDTA and ^{24}Na in three successive tracer injection tests in each of three experiments before during and after reversal of induced edema development* Isolated plasma perfused rabbit lung preparations The evaluated extracted amounts for ^{51}CrEDTA and ^{24}Na were determined as described in the text and in Fig 2 and plotted in same arbitrary units as in Fig 3 Test no 1 in each experiment is a control test The second test in each experiment was carried out during the development of edema induced by adding EDTA to the perfusate (see text) The third test in each experiment was carried out after reversal of edema development and reduction in preparation weight to the level seen before EDTA addition (see text and Methods)

■——■
□- - -□
▲——▲
△- - -△
●——●
○- - -○

ated extracted amount' in the second test less than the mean of the values for the first and the third tests (Fig 4) It will also be seen from Fig 4 that the individual values of "evaluated extracted amounts" varied considerably in these tests

The effect of changes in perfusate flow on the "evaluated extracted amounts" of diffusible tracers If the exchange across the walls of the exchange vessels during one single passage of diffusible tracers is limited by the rate of diffusion out of the vessels, then an increase in the contact time between the tracers and the vascular bed

should increase the degree of exchange In three experiments an alteration in this contact time was attempted by variations in the flow through the preparation In one of these experiments ^{24}Na was the only diffusible tracer used The first and the third tracer injection tests were performed in situations of normal flow, whereas the second one was performed in a situation of halved flow (see Methods) Such a reduction in flow resulted in a 30 % lowering of the pulmonary arterial pressure Changes in preparation weight were also seen when the perfusate flow was changed However, the injection tests were carried out at conditions of stable preparation weight.

In Fig 5 is shown the "evaluated extracted amounts" of diffusible tracers from the tests performed in this group of experiments In two of the experiments this parameter had a larger value in the second test than in the first and third one In the third experiment of this group no definite trend of this type could be observed It should nevertheless be noted that in all cases but one was the "evaluated extracted amount" larger in the third test than in the first one

"Evaluated extracted amounts" of the diffusible tracers in tests performed during development of EDTA induced edema During development in this preparation of EDTA induced edema capillary permeability to proteins is most probably increased (Nicolaysen 1971 a) One would expect then that also the permeability to smaller solute molecules was increased in this situation If the exchange of diffusible tracers during one single passage through the organ is diffusion limited in a normal preparation, then one would expect that a larger extraction of such tracers should occur in tests carried out during the EDTA induced edema development

Development of edema by addition of EDTA was induced in three experiments (see Methods) A first tracer injection test was performed during an ordinary perfusion period The second tracer injection test was performed when the preparation had gained 1 5—1 8 g in weight The rate of the weight increase at the moment of this second test was 0 10—0 15 g/min The third tracer injection test was performed when the edema formation had been stopped by $CaCl_2$-addition and the weight had declined to a level similar to that seen before the EDTA addition (see Methods for detailed description of experimental procedure)

The 'evaluated extracted amounts' from the tests performed in these experiments are shown in Fig 6 The evaluated extracted amount' in the second test was each time higher than the mean of the values in the first and in the third test of the same experiment In addition the evaluated extracted amount was consistently larger in the third test than in the first one of the same experiment (Fig 6)

Discussion

A common finding in all the tracer injection tests performed in the present experiments was that in the early outflow fractions where the concentrations of the tracers were increasing the normalized concentration of ^{24}Na was lower than that of ^{51}CrEDTA which again was lower than that of ^{125}I albumin (Fig 1) These

concentration patterns of the tracers in the outflow fractions seem to indicate that some diffusion of ^{24}Na and $^{51}CrEDTA$ out of the vascular bed took place initially An outflow separation of this type could also have been caused by interlaminar diffusion, the so called Taylor effect (Taylor 1953, for further references see Lassen and Crone 1970) There are, however, several arguments against the Taylor effect being the sole explanation for the initial apparent extraction of small tracer molecules Firstly, it was a consistent finding that the fractional extraction of $^{51}CrEDTA$ reached the zero level in an earlier fraction than did that of ^{24}Na (Fig 1, upper part) Also the two curves for fractional extraction of ^{24}Na and $^{51}CrEDTA$ were almost parallel (Fig 1, upper part) If interlaminar diffusion alone should have caused "extraction" of these tracers, then one would expect, on the basis of the greater rate of diffusion of ^{24}Na, the curve for this ion to be the steeper one and also the fractional extraction curve for ^{24}Na to reach the zero level earlier than that for $^{51}CrEDTA$ Another argument stems from observations on the "evaluated extracted amounts" This parameter reflects the accumulated loss of diffusible tracer from all outflow fractions with lower concentrations of diffusible tracers than of reference tracer In all the control experiments the "evaluated extracted amounts" of ^{24}Na as well as of $^{51}CrEDTA$ were larger in the third tracer injection test than in the first one In the experiments with flow variation and in those with induced edema formation the experimental conditions in the first and third tests were presumed to be the same Here too the "evaluated extracted amounts" of the two tracer ions were larger in the third than in the first test in 10 out of 11 instances Such changes from one test to another and similarly performed test can hardly be explained on the basis of a Taylor effect It seems reasonable to infer, therefore, that some transvascular passage of the diffusible tracers did take place in the present experiments during one single passage of the indicators through the vascular bed The possibility of some diffusion out of the vascular bed also of ^{125}I-albumin during one single tracer passage was not tested in the present experiments

A rapidly declining fractional extraction of diffusible tracers was observed in the phase of increasing intravascular concentrations of tracers in the present experiments (Fig 1, upper part) This indicates that the concentrations of these tracers have probably increased rapidly in the extravascular space The concentration gradient for the diffusible tracers will then not be proportional to the intravascular concentrations The extravascular space of distribution has thus most probably been an important limiting factor for the amount of diffusible tracers leaving the vascular bed during the tests in the present experiments

This conclusion is not in agreement with that of Bauman *et al* (1957) and of Chinard, Enns and Nolan (1962) Bauman *et al* (1957) seemed to observe quite stable fractional extractions of ^{24}Na in the early outflow fractions after single injections of tracers into the pulmonary circulation of healthy humans The discrepancy here might be due to species differences between human and rabbit lungs as regards available extravascular space for indicator distribution

The fractional extraction of the larger diffusible tracer, $^{51}CrEDTA$, reached the

zero level earlier than did the fractional extraction of the smaller diffusible tracer ^{24}Na If the same extravascular space of distribution had been available for these two tracers then one would expect the fractional extraction of ^{24}Na to decline along a steeper curve and to reach zero in an earlier fraction than the larger molecule ^{51}CrEDTA The most probable explanation for this not being the case is that the two tracers had different volumes of distribution It is thus possible that the ^{24}Na could exchange also with some intracellular sodium, possibly in endothelial cells The ^{51}CrEDTA anion is about the size of the sucrose molecule and does probably not enter cells to any measurable extent during the short time of contact prevailing in the present tests

In the two control experiments six tests were performed with in all twelve determinations of "evaluated extracted amounts" for ^{24}Na or ^{51}CrEDTA Only moderate changes in each set of these calculated values occurred in the course of any one of these experiments This indicates a relatively high degree of reproducibility with this method in the present preparation It was felt therefore that a more marked change in the value for "evaluated extracted amount" occurring in one and the same experiment could be regarded as reflecting a real functional change in the preparation

The results from the experiments where the extravascular fluid volume was expanded through filtration, are apparently contradictory to the conclusion that the extravascular space of distribution constitutes an important limiting factor for diffusible tracer exchange during one single tracer passage In only one out of six cases was the "evaluated extracted amount" value largest in the situation with increased extravascular fluid volume The three sodium values were all reduced in this situation This unexpected finding is not easy to explain The increase in extravascular volume of about 1 5 g was a considerable one, since the wet weight of the lung tissue proper in this preparation is about 7 g (Lunde 1967) It is possible that the fluid filtered out from the vessels has not been fully available for exchange with the intravascularly injected diffusible tracers In another series of experiments with the same lung preparation ultrastructural studies were carried out after perfusion fixation during hydrostatically induced capillary filtration (Hovig Nicolaysen and Nicolaysen 1971) These studies indicated that for large sections of the alveolar capillaries only small increases in extravascular fluid had taken place in the immediate capillary wall vicinity

Also the first and third injection tests in the present "filtration experiments" were carried out at conditions of increased capillary pressure It may be that the increment in extravascular fluid volume which becomes available for the diffusible tracers is small and takes place quickly when capillary pressure is elevated The fact that the "evaluated extracted amount" of ^{24}Na in the first tests of these experiments was larger than in the first tests performed in the other groups of experiments, could indicate that this was the case

West Dollery and Heard (1965) and later Ritchie Schauberger and Staub (1969) have described changes in flow distribution with a reduced flow in the lower pulmonary sections, in isolated perfused lungs during periods with elevated out

flow pressure In experiments of the present type such changes in flow distribution *could imply changes in area available for* exchange, length of contact time and possibly also changes in degree of interlaminar diffusion It is not known, and there was no easy way of finding out, whether such changes in flow distribution did take place during periods with elevated outflow pressure in the present experiments However, several important parameters other than extravascular volume may have changed during these experiments

In the experiments with variations in perfusate flow the reduction in flow may not have resulted in the expected increase in contact time between the tracers and the walls of the exchange vessels The number of perfused capillaries could have been reduced in the periods of reduced flow, due to the reduced inflow pressure A reduction in number of perfused capillaries would result in both a reduced area for exchange and a smaller increase in contact time than theoretically possible Furthermore, the extravascular fluid space could be somewhat reduced as the result of the reduced intravascular pressures Two of these flow-experiments showed increased "evaluated extracted amounts" in the situation of reduced flow (Fig 5) This set of results is consistent with there being some transvascular diffusion limitation for smaller solute molecules at large and rapid perfusate flows through the present preparation Interfering, oppositely acting factors may have cancelled out an effect of an increased contact time in the third experiment in this group

The inference that the extravascular space of distribution is limiting for the early extraction of small solute molecules and ions during one single passage, does not leave much room for discovering alterations in the capillary permeability to ' tracers However all the experiments in which edema formation was induced showed a definite increase in 'evaluated extracted amount" of ^{24}Na as well as of $^{51}CrEDTA$ during the edema development (Fig 6) True, the extravascular space available for solute exchange was possibly larger in the situation with edema formation than in the control tests in the same experiment Although the role of the increased extravascular volume is somewhat unclear, it seems likely that some of the increases in "evaluated extracted amounts' observed during edema development reflect an increased capillary permeability present at that time

Capillary permeability to albumin was most probably increased during the development of EDTA induced edema In that situation the ^{125}I albumin may therefore not be a good intravascular tracer The real increase in extraction of the smaller solute molecules in the edema situation may thus have been larger than evaluated in the present experiments

A moderate increase in the "evaluated extracted amounts' seemed to occur with time from start of perfusion in most of the experiments The weight development of the preparations did not indicate that any increase in extravascular fluid volume had taken place Nor were there any indications of an increase with time of perfusion in the number of perfused capillaries Thus this increase in "evaluated extracted amounts" was probably due to some increase in capillary permeability with time of perfusion This, together with the findings from the edema experiments, indicate

that there has been some diffusion limitation to smaller solute molecules and ions under the conditions prevailing in the present preparation Extensive alterations in permeability, such as is probably present during EDTA induced edema formation could just be detected by the application of the indicator diffusion method It appears improbable, though that accurate information on more moderate changes in capil lary permeability in this rabbit lung preparation can be detected with this method in its present form

I want to thank Miss I K Nordby for skilful technical assistance and Mr T Eriksen and his staff for construction and development of some of the instruments used in the study This work has been supported by grants to the Institute of Physiology from Hjelpestikkene , The Nansen Foundation and The Norwegian Research Council for Science and the Humanities The powdered heparin was generously supplied by Novo Industri A/S Oslo and Copenhagen

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From the D... of Physiology, University of Göteborg, Sweden

A Note on Knee-joint Denervation and Postural Reflexes in the Cat

By

... LINDSTRÖM and ULF NORRSELL

Evidence has been p... icating that receptors located in the joints are important for man's c... c... reciation of the position of a limb (Merton 1964). Afferent nervous a... ... ceptors located in a joint has also been found to influence the transm... ... reflex arcs (Skoglund 1956), and pathways from joint afferents t... ... have been demonstrated (Hongo, Jankowska and Lundberg 1969 ... with regard to the cat. Thus joint receptors could be involved in fun... ... from lower motor regulation to those at the highest level. Accordingl... ... that partial denervation of a joint caused changes in postural refl... (Freeman and Wyke 1966, Wyke 1967) was of considerable interest.

Freeman and Wyke (1966) found that transection of the posterior articular nerve in the cat caused reproducible changes in what they described as 'reflex behaviour' as well as a number of reflexes specifically related to the ... of the hindlimb musculature. The reflex behaviour altered by the ne... was stance, gait, jumping and bar walking. The specific reflexes altered ... ectomy ... the suspension (elevation) reflex, the Stutz ... reaction. Transection of the posterior ... nervation of the knee joint of that limb ... partial denervation of the knee joint ... lar nerve.

We have repeated Freeman and ... the posterior articular nerve in one ... ever, when the cat 48 hrs after th... anesthesia (Nembutal Abbot 50 ... observed. The posture of the a... sitting or standing still were qui... when the cat was jumping up ... on the floor or on a 48 mm ... normal. There was no differenc... Neither was any difference b... pended vertically nor when tes...

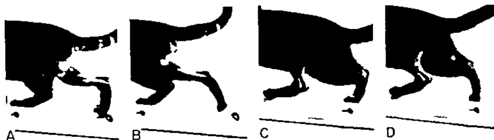


Fig 1 Cinematographic record of a cat walking on a 48 mm wide bar 72 hours after denervation of the left knee joint. For full explanation see text

that no postoperative effects were going to be seen, at least not with the above methods, the animal was sacrificed 16 days after the operation and both hindlimbs dissected carefully Nothing was found to indicate that any other lesion had been made than the one intended

Because of this unexpected finding we then tried the effect of a more extensive knee joint denervation Thus in 2 cats not only the posterior but also the medial articular nerve was transected in one hindlimb in the course of one operation, as well as those branches of the lateral articular nerves, which could be approached without too much manipulation of the common peroneal nerve In neither cat did we observe any of the effects of knee-joint denervation described by Freeman and Wyke (1966) Of these effects, listed above, the most distinct one concerned bar walking, which the animals were described as reluctant to make for the first 10—14 days after operation When the animal was placed on the beam 'he crouches with his belly pressed to its upper surface and grips it tightly etc ' The pictures in Fig 1 derive from a cinematographic recording of a cat walking on a 48 mm wide bar 72 hrs after transection of the posterior and medial as well as part of the lateral articular nerve in the left hindlimb Picture A was taken less than 0 06 sec after the cat had placed the right (control) hindlimb on the bar B was taken 0 2 sec after A when the cat had started lifting the left operated limb C was taken 0 25 sec after B and less than 0 06 sec after the cat had placed the left operated limb on the bar D was taken 0 2 sec after C when the cat started lifting the right (control) limb As evident from these pictures the cat was moving rapidly and there was no difference between the operated and unoperated limb The angles at which the cat placed its hindlimbs, which is a point stressed by Freeman and Wyke (1966) are those used by a normal cat under these circumstances (vide also Engberg and Lundberg 1969) The cat of the figure was taken to acute experiment 8 days later and the transected nerves the distal parts of which had been marked with ligatures followed to the joint capsule As an extra control the same nerves of the other limb were dissected and found equal and their status as afferent joint nerves was verified electrophysiologically

Thus, having failed to reproduce any of the results described by Freeman and Wyke (1966) we conclude that these author's results, whatever their cause, were not necessarily due to denervation of the knee-joint. Perhaps conditioning techniques will provide a better approach for evaluating the functional significance of the afferent nervous activity from the joints of animals (vide Ovsiannikov 1967).

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Acta physiol scand. 1971. 82. 409—411

From the Brain Research Laboratory, E-blocket the University Hospital of Lund and from the Department of Surgery, University of Lund, Sweden

Cerebral Blood Flow and Cerebral Energy State

By

Bo Eklöf and Bo K Siesjö

Although vigorous hyperventilation can reduce the overall cerebral blood flow to 45—65 % of normal (Kety and Schmidt 1946, Reivich 1964, Wollman *et al* 1968) without causing any serious disturbances of cerebral energy metabolism (Alexander *et al* 1968, Granholm and Siesjö 1971), it is not known how far the blood flow can be reduced before the energy state of the tissue is severely affected This is mostly due to the fact that there is a very narrow margin between a reduction in flow which partially inhibits energy production in the tissue, and a flow which is in sufficient for the survival of the organism

In order to study the quantitative relation between the cerebral blood flow and the energy state of the tissue we have ligated both carotid arteries in lightly anesthetized (30 % O_2—70 % N_2O) rats, either at normal mean arterial blood pressure or after a reduction of the pressure by means of bleeding to 100 or 70 mm Hg, respectively The energy state of the tissue was evaluated from the tissue contents of labile phosphates (phosphocreatine, ATP, ADP and AMP) and of substrate couples participating in redox reactions (lactate and pyruvate) after freezing the tissue *in situ* (see Siesjö and Nilsson 1971) Since the parameters which are necessary for a calculation of intracellular pH were determined it was possible to derive cytoplasmatic $NADH/NAD^+$ ratios and to evaluate the part played by acidosis in shifting the creatine phosphokinase reaction in the direction of decreased phosphocreatine levels (Siesjö and Messeter 1971 Messeter and Siesjö 1971) In lack of a quantitative method for measurements of the cerebral blood flow in the rat we have estimated the relative decreases in flow from the arteriovenous carbon dioxide and oxygen differences (femoral artery and superior sagittal sinus) on the assumption of an unchanged cerebral metabolic rate This was done by measuring the arteriovenous differences for carbon dioxide by means of a microdiffusion method and by calculating the oxygen differences from the P_{O_2} pH and hemoglobin values using a rat oxygen dissociation curve

The table shows the arterial and venous oxygen tensions, the tissue contents of phosphocreatine ATP ADP and AMP and the calculated cytoplasmatic NADH NAD ratios in one control group with loose ligatures around the carotid arteries

TABLE I. [illegible]

	P_{aO_2}	P_{vO_2}	PCr	ATP	ADP	AMP	Lact	NADH/ NAD+
Control	120	51 5	4 89	2 85	0 42	0 04	1 68	2 60
	±2 7	±1 3	±0 1	±0 04	±0 03	±0 002	±0 05	±0 23
Carot lig	123	36 2	4 59	2 73	0 39	0 04	3 15	2 60
MABP = 160	±4 1	±1 2	±0 1	±0 12	±0 02	±0 004	±0 96	±0 45
Carot lig	130	32 9	2 92	2 08	0 65	0 41	20 47	9 86
MABP = 100	±4 1	±1 7	±0 7	±0 34	±0 11	±0 18	±6 34	±2 9

and in two groups studied 30 min after bilateral carotid artery ligation In the first of these ligated groups the blood pressure was allowed to attain its spontaneous value, but in the second the mean arterial blood pressure was reduced to, and kept at, 100 mm Hg

Carotid artery ligation alone did not lead to signs of a serious disturbance of the cerebral energy state, and it should be stressed that neither the cytoplasmatic NADH/NAD ratio nor the energy charge potential, defined according to Atkinson (1968) as [(ATP) + 0 5 (ADP)]/[(ATP) + (ADP) + (AMP)] was significantly different from the controls The energy state was preserved in spite of the fact that the venous P_{O_2} was reduced by about 10 mm Hg, and that the decrease in flow, calculated from the CO_2 and O_2 differences, were 46 and 49 %, respectively, of the normal

When the carotid arteries were ligated at a mean arterial blood pressure of 100 mm Hg there were large changes in phosphocreatine, ATP, ADP, and AMP, as well as in the NADH/NAD+ ratio These changes occurred at a venous P_{O_2} of 33 mm Hg and at a reduction in flow to below 45 % of the normal, as calculated from A—V oxygen differences Even if the method will underestimate the reduction in flow if the cerebral metabolic rate is decreased, we may tentatively conclude that a reduction in cerebral blood flow to below about 45 % of the normal value leads to a disturbed tissue energy metabolism and thus to an unbalance between energy production and energy utilization in the tissue However, even if the change in the energy state of the tissue may be related to the decrease in tissue perfusion, an accompanying communication (MacMillan and Siesjo, this volume) will show that this change cannot be related to the lowering of the sagittal sinus P_{O_2} from 52 to 33 mm Hg

This study was supported by grants from the Swedish Medical Research Council (Project No B71-14X-263-07A and B71 14X 2179-03), from the Swedish Bank Tercentenary Fund, and by U S PHS Grant No 5 RO1 NS 07838 02 from NIH

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Acta physiol. scand. 1971. 82. 412—414

From the Brain Research Laboratory, E blocket, the University Hospital of Lund, Sweden

Critical Oxygen Tensions in the Brain

By

VERNON MACMILLAN[1] and BO K SIESJO

The brain has a relatively sparse capillarity and a high resting oxygen consumption and it therefore relies on a continuous supply of oxygen The force determining the rate of supply is the P_{O_2} gradient between capillary blood and tissue Since isolated mitochondria can respire maximally down to, or even below, P_{O_2} s of 1—3 mm Hg (Chance *et al* 1962, Lubbers and Kessler 1968) an undisturbed oxidative phosphorylation would require a P_{O_2} gradient sufficient to maintain these P_{O_2} values in all tissue areas Theoretical calculations on diffusion models predict that the brain is normally just sufficiently supplied with oxygen, and that decreases in the capillary and venous P_{O_2}'s, from whatever cause, lead to tissue hypoxia (Thews 1963, Grote 1967) Although the actual tissue capillary arrangement may allow a better oxygenation than that predicted by the conventional models (Lübbers 1968 Grunewald 1968), the models fit with the observations that consciousness is lost at a venous P_{O_2} of 17—19 mm Hg ("critical threshold", see Opitz and Schneider 1950) and that lowering of the venous P_{O_2} to 12—14 mm Hg leads to such severe hypoxia that death of the organism threatens ("deadly threshold")

In the rat, which normally has a high venous P_{O_2} (about 50 mm Hg), a reduction of cerebral blood flow to below about 45 % of normal leads to a reduction of the venous P_{O_2} to 30—35 mm Hg and to changes in the energy state of the tissue, suggestive of a serious disturbance of the oxidative metabolism (Eklof and Siesjo, this journal) If the difference in the positions of the oxygen dissociation curve of rat and man are taken into account this degree of desoxygenation of cerebral venous blood is found to correspond to a P_{O_2} of about 20 mm Hg in man, *i e* to values close to the "critical threshold" However, as will be shown, it can be seriously questioned if the venous P_{O_2} has the assumed relevance for the problem of oxgen diffusion to the tissue

The experiments involved a 30 minute reduction of arterial P_{O_2} in lightly anesthetized normotensive and normocapnic rats with measurements of arterial and superior sagittal sinus P_{O_2} and of the tissue contents of labile phosphates plus lactate and pyruvate (see Siesjo and Nilsson 1971) For the whole P_{O_2} range, the intracellular lactate and pyruvate concentrations as well as the intracellular pH' were determined, thus allowing calculation of cytoplasmatic $NADH/NAD^+$ ratios

[1] R S McLaughlin fellow on leave of absence from the Department of medicine (Neurology), Toronto General Hospital, Toronto Canada

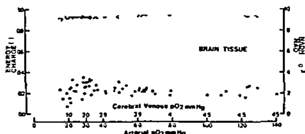


Fig 1 Energy charge potential of brain adenine nucleotides (ATP+ +0 5×ADP) (ATP+ADP+AMP) and cytoplasmatic NADH /NAD$^+$ ratio related to arterial and cerebral venous oxygen tensions in animals subjected to a 30 min decrease in the arterial O_2 tension Note absence of changes in energy charge potential or in NADH/NAD$^+$ ratio in spite of cerebral venous pO_2 s of 10 mm Hg

As in the previous report (Siesjo and Nilsson 1971), reduction of the arterial P_{O_2} below 50 mm Hg gave rise to a progressive tissue lactacidosis and to a decrease in the phosphocreatine content However, the fall in the phosphocreatine content was not larger than what could be expected from a pH dependent shift in the creatine phosphokinase equilibrium (see Kuby and Noltman 1963, Siesjo and Messeter 1971) Moreover, the arterial P_{O_2} could be reduced to below 20 mm Hg without any significant changes in the tissue contents of ATP, ADP or AMP, and without any increase in the cytoplasmatic NADH/NAD$^+$ ratio In order to evaluate the presence of any disbalance between the rate of utilization and the rate of production of ATP the energy charge of the adenine nucleotide pool—(ATP+0 5 ADP)/(ATP+ADP+ +AMP) — was calculated (Atkinson 1968) Fig 1 shows that both the energy charge ratio and the calculated NADH/NAD$^+$ ratio remained essentially unchanged down to P_{O_2} values of 15—16 mm Hg corresponding to cerebral venous P_{O_2} values of about 10 mm Hg Lower O_2 tensions could not be studied since they were invariably associated with a fall in blood pressure, and then with changes in the energy charge ratio (*cf* Siesjo and Nilsson 1971)

The implication of the present results is apparent if it is recalled that a reduction of the cerebral blood flow large enough to lower the venous P_{O_2} to 33 mm Hg is accompanied by a fall in the energy charge ratio to 0 7 and by a large increase in the NADH/NAD$^+$ ratio (Eklof and Siesjo, this volume) In the present experiments, which involved reduction of the arterial P_{O_2} at normal blood pressure (120 mm Hg), a lowering of the venous P_{O_2} to about 10 mm Hg gave no such changes The results suggest that the brain mitochondria may be sufficiently supplied with oxygen even when the driving capillary tissue P_{O_2} gradient is of the order of 10 mm Hg, and that conventional capillary models may not suffice to describe oxygen diffusion in the brain It is further suggested that the venous oxygen tension observed during a fall in the cerebral perfusion pressure is poorly related to the actual capillary P_{O_2} which must exist in parts of the tissue In other words, the change in the energy state of the tissue elicited by a fall in perfusion pressure must be due to the fact that some capillaries are so poorly perfused that the end-capillary P_{O_2} falls well below 10 mm Hg, while others may be normally perfused It therefore seems likely that a degree of

cerebral hypoxia which affects the energy state of the tissue almost always is synonymous with regional ischemia, and it appears fruitful to direct research on the pathogenesis of irreversible hypoxic brain damage towards the capillary circulation

This study was supported by grants from the Swedish Medical Research Council (Project No B71-14X-263-07A and B71-14X-2179 03), from the Swedish Bank Tercentenary Fund, and by U S PHS Grant No 5 R01 NS 07838 02 from NIH.

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Acta physiol. scand 1971. 82. 415—416

From the Obstetric Clinic, Karolinska Sjukhuset, Stockholm, Sweden

Induction of Midtrimester Abortion by Intra-Amniotic Administration of Prostaglandin $F_{2\alpha}$

A Preliminary Report

By

M BYGDEMAN, M TOPPOZADA and N WIQVIST

Continuous i v infusion of prostaglandin (PG) has proved to be reasonably successful for the induction of abortion (Bygdeman and Wiqvist 1970, Karim and Filshie 1970, Roth Brandel *et al* 1970) However, the general opinion of most investigators is that efficient doses are associated with a high incidence of side effects, such as vomiting diarrhea and local erythema at the site of venepuncture

Intra uterine administration of PG via a catheter between the fetal membranes and the uterine wall is associated with a significantly lower incidence of side effects This route of administration probably allows a direct action of PG on the myometrium, thus obviating the necessity for a high systemic concentration Occasionally, however, the drug may reach the systemic circulation by a direct passage into the effluent uterine veins, this may precipitate side effects and also reduce the efficiency of the injected dose (Wiqvist and Bygdeman 1970)

The aim of this study was to investigate another local route of administration by intra amniotic injection of PG, thereby eliminating the risk of i v leakage and providing a better distribution of the drug to the uterine wall 9 women in the 14th—20th week of gestation admitted to the hospital for induction of legal abortion volunteered for the investigation Intermittent injections of a $PGF_{2\alpha}$ solution (5 mg/ml) were given through a polyethylene catheter introduced into the amniotic cavity by abdominal puncture The same catheter was utilized for recording of amniotic pressure Separate doses ranging between 5—15 mg $PGF_{2\alpha}$ were administered at intervals of 3—14 h depending on the contractile response of the uterus It was found that on intra amniotic injection of 5—15 mg $PGF_{2\alpha}$ the uterus responded within 60 min with a gradual increment of contractile activity characterized by a rapid increase in the frequency of the contractions The amplitude of the contractions, however increased gradually throughout the course of administration to become effective and fairly regular within a period of approximately 6 h with an elevation of uterine tone in 5 out of the 9 recorded cases

It was possible to induce abortion in all 9 women without any failures The last 3 cases indicate that 1 or 2 injections might be sufficient to evacuate the uterus 4 of the abortions were complete and the remaining 5 were incomplete with a

TABLE I Induction of abortion by intra amniotic injection of prostaglandin $F_{2\alpha}$

Case	Week of pregnancy	Number of injections	Total dose (mg)	Interval 1st injection — Abortion (h)	Side effects	Outcome
1	18	12	124 0	51	0	Incomplete abortion
2	14	5	45 0	30	0	Incomplete abortion
3	18	8	37 0	24	vomiting (6 episodes)	Incomplete abortion
4	19	7	32 0	21	0	Complete abortion
5	14	6	30 0	24	0	Incomplete abortion
6	18	3	25 0	14	0	Complete abortion
7	18	2	20,0	20	0	Complete abortion
8	14	2	14 0	12*	0	Incomplete abortion
9	15	1	10 0	25	vomiting (3 episodes)	Complete abortion

* Spontaneous rupture of the membranes 1 5 h following the first injection

retained placenta following expulsion of the fetus (Table I) Intra amniotic administration of $PGF_{2\alpha}$ was not associated with any generalized side effects except for two patients who had some episodes of vomiting These patients experienced pain which might have been the precipitating factor for the vomiting

In a previous report (Wiqvist *et al* 1968), the intra-amniotic injection of low oses of PGE_1 (in the order of 75 μg) at mid-pregnancy, had no appreciable effect on uterine contractility However, the lack of response was probably due to the fact that too low doses were employed

As judged from the uterine contractility recordings as well as the infrequent occurrence of generalized side effects, it is believed that intra-amniotically injected $PGF_{2\alpha}$ acts locally on the myometrium after diffusion through the fetal membranes In this way, the drug is distributed over a large surface area of the myometrium providing a smooth and efficient stimulation

From this preliminary report of a new route of PG administration it is our impression that it is an efficient, safe and easily tolerated procedure for the induction of second trimester abortion

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Acta physiol. scand 1971. 82. 417—432

From the University Institute of Pathological Anatomy, Laboratory of Electron Microscopy, Rikshospitalet and Institute of Physiology, University of Oslo Norway

Ultrastructural Studies of the Alveolar-Capillary Barrier in Isolated Plasma-Perfused Rabbit Lungs. Effects of EDTA and of Increased Capillary Pressure

By

T Hovig, Anne Nicolaysen and G Nicolaysen

Received 8 January 1971

Abstract

Hovig, T, A Nicolaysen and G Nicolaysen *Ultrastructural studies of the alveolar capillary barrier in isolated plasma perfused rabbit lungs Effects of EDTA and of increased capillary pressure* Acta physiol scand 1971 *82* 417—432

The ultrastructure of lung capillaries and of lung interstitium has been studied in isolated ventilated and plasma perfused rabbit lung preparations at different levels of perfusate [Ca^{++}] and [Mg] (achieved by EDTA additions) A perfusion fixation method was applied and situations where net fluid transfer over the capillary wall was taking place could therefore be [illegible]

(laminae rarae) of the basement membranes which were much more distinct than in control specimens This phenomenon could be related to the process of capillary fluid leakage which thus may take place also in the regions of the vessel wall where the alveolar-capillary barrier is at its thinnest

Changes in the permeability of the capillaries in isolated perfused lungs have been shown to occur on reductions in intravascular concentrations of ionized calcium and ionized magnesium (Nicolaysen 1971 a and b) Such reductions were achieved by addition to the perfusate of the chelators ethylenediaminetetraacetic acid (EDTA) or ethyleneglycoldiaminetetraacetic acid (EGTA Edema developed when at the same time the concentrations of both Ca^{++} and Mg were reduced to very low values by addition of EDTA (Nicolaysen 1971 a) An increase in the hydraulic conductivity of the exchange vessels (capillaries was observed when a much smaller reduction in [Ca] was established by appropriate EDTA or EGTA additions (Nicolaysen 1971 b) Mg could *not* substitute for Ca in this situation A possible explanation for these findings is that the capillary wall was affected at one site on intravascular

reductions in [Ca⁺⁺] and at another site when [Ca⁺⁺] as well as [Mg⁺⁺] were markedly reduced

There has not been much information available on ultrastructural changes in capillaries exposed to low perfusate [Ca⁺⁺] and/or [Mg⁺⁺] Clementi and Palade (1969) exposed intestinal capillaries for 10 min to a salt solution perfusate containing EDTA They observed changes in the capillaries only at concentrations of EDTA much higher than those applied by Nicolaysen (1971 a and b) Clementi and Palade made no attempts to correlate their findings with measurements of capillary permeability The difference in concentrations of EDTA applied and the difference in organ under study make it difficult to compare their findings and those made in the present lung preparation

Using light microscopy, Staub, Nagano and Pearce (1967) studied the sequence of fluid accumulation in lungs during edema formation They found that fluid initially accumulated in the interstitial connective tissue compartments around the larger blood vessels and airways The fluid leakage during edema formation may possibly take place from the alveolar capillaries proper but the pathways to the larger interstitial compartments, however, have not been distinctly demonstrated

The aim of the present work was to study the ultrastructure of the pulmonary capillary barrier at EDTA induced edema formation as well as at EDTA induced increase in capillary hydraulic conductivity Furthermore we tried to get information on the extravascular tissue changes during induced edema formation The fixative (glutaraldehyde) was suddenly applied to the vascular bed at unchanged intravascular and intraalveolar pressures without any interruption of the perfusion

The morphological observations revealed no specific changes in the capillary barrier which could explain the EDTA induced edema development or the increase in hydraulic conductivity However the electron lucent zones (laminae rarae) of the basement membranes were definitely more distinct in sections from preparations fixed during edema development than in sections from control preparations The same phenomenon was observed although to a lesser extent in sections from preparations fixed during outward capillary filtration at normal as well as at somewhat reduced [Ca⁺⁺] and [Mg⁺⁺]

Materials and Methods

[illegible] tone [illegible]

glass cannulas were placed in the left auricle and through the right ventricle into the pulmonary artery

The isolated lungs were perfused under conditions of constant volume pulsatile inflow per [illegible] started 9—14 min after the rabbit's own circulation had been stopped Both lungs [illegible] mean [illegible] d not [illegible] Im-mediately after the start of perfusion the lungs [illegible] ventilation with a mixture of 5 % CO_2 in air was started A positive [illegible] pressure of 10 cm of water and an end expiratory pressure of 2 cm of water was used The

pulmonary arterial pressure was continuously recorded (Sanborn P23Db pressure transducer) Weight changes of the preparation were continuously recorded by having the preparation

resistance during perfusion

About 1 h before start of an experiment a plasma portion was thawed and filtered once through one layer of filter paper

A more detailed description of this experimental arrangement has been given earlier (Nicolaysen 1971 a)

Perfusion fixation method The lung tissue fixation procedure was carried out in the fol

Krebs Ringer Dextran solution

s solution contained in mmoles

1 2 Each liter also contained

min after start of the perfusate exchange the ventilation was stopped during inspiration by clamping the trachea Immediately afterwards perfusion with a solution containing 1 7 % glutaraldehyde in 0 08 M phosphate

solution was immediately adjusted so that the pulmonary arterial pressure remained unchanged (flow about 350—400 ml/min) The first portion of fluid flowing out from the pulmonary vascular bed after the change to glutaraldehyde perfusion was discarded in order to avoid contamination of the fixation solution The perfusion fixation lasted for 15—20 min The lungs developed a uniform yellow tinge after only a few min The intermediate perfusion with the

examined in a Siemens Elmiskop I electron microscope

Results

Control experiments

Since the ultrastructure of isolated plasma perfused and ventilated rabbit lungs has not been described, two pairs of lungs were perfused with plasma for 20—30 min, and the perfusion fixation then carried out as described in Methods The weight of the preparations remained unchanged during the fixation procedure

By *light microscopic orientation* on semi thin sections the pulmonary microcirculatory vessels appeared empty (Fig 1) Only rarely was any blood corpuscle observed indicating that an efficient perfusion of the pulmonary vascular bed had taken place The alveoli had a somewhat irregular outline, partly because capillaries were bulging into them The alveoli appeared empty (Fig 1) except for the presence of occasional macrophages

Ultrastructural studies The alveolar capillaries were found to be of the continuous type The endothelium contained organelles which were concentrated in

the perikaryon as has been described by Weibel (1969) Mitochondria, ergastoplasm Golgi apparatus and some microtubuli were observed (Fig 3 and 5) all with a normal appearance The endothelium contained numerous pinocytotic vesicles The nuclear envelope had a normal appearance The chromatin of the nuclei of the endothelial cells was distributed around the periphery with a few clumps more centrally located (Fig 3)

The intercellular clefts in the endothelium were in some instances short, running straight from the luminal side to the side of the basement membrane (Fig 5) In most instances, however, they showed a more or less tortuous course Some inter cellular junctions appeared to be open (Fig 5) The problem of whether these junctions were really open or not was not studied in detail with for instance block staining with uranyl acetate, serial sections or specimen tilting

In the thinnest parts of the alveolar-capillary barrier the basement membranes of the endothelium and of the epithelium appeared fused making a nearly homogenous zone between the endothelium and the epithelium (Fig 4 and 5) In some instances narrow, less electron dense zones (laminae rarae) of the basement membranes could be discerned close to the endo- and epithelium

The epithelial lining of the alveoli was composed of the two types of cells the membraneous and the granular pneumocytes as has earlier been described (*e g* Weibel 1969) In places the alveolar epithelium was covered by a thin extracellular lining layer most probably representing the surfactant (Weibel and Gil 1968 Gil and Weibel 1969/70) In some instances this lining appeared as membranes or tubules (Fig 9) as described by Gil and Weibel (1969/70)

The minimum sized alveolar-capillary interstitium in the lungs consisted of the apparently fused basement membranes of epi and endothelium (Fig 4) The minimum thickness observed over any length of the alveolar-capillary barrier was about 0 1 μ but at sites of membrane infoldings (pinocytotic vesicles) the distance could be as small as 300—400 Å In other places a wider interstitial space containing collagen fibrils elastic tissue and interstitial cells was observed Fig 3 shows a part of such a wider section of the alveolar capillary barrier Around terminal branches of the airways and around small arteries and veins the interstitium was somewhat more abundant

From the general appearance of the endothelial and epithelial cells and of the organelles in these cells it seems justified to conclude that the tissue was well preserved

Lungs fixed during EDTA induced edema formation

In 2 expts the lungs were initially perfused with plasma for 20—30 min next EDTA (67 mM in saline) was added The concentration of EDTA in the perfusate was adjusted to about 4 3 mM corresponding to 120—130 % of the sum of the concentrations of total calcium Ca_t (ionized plus non ionized calcium) and total magnesium, Mg_t, in the plasma Such a concentration of EDTA is known to result in edema development in this lung preparation (Nicolaysen 1971 a) As expected an

accelerating increase in preparation weight was observed after about 20 min The pulmonary arterial pressure remained unchanged When the preparations had gained 1 5—2 0 g in weight, the KRD perfusion was started In the KRD the concentration of EDTA relative to the concentration of calcium and magnesium was the same as in the plasma During this KRD perfusion the accelerating increase in weight continued The preparations had gained a total of about 3 5—4 g in weight from the moment of the EDTA addition to the start of the glutaraldehyde perfusion The accelerating increase in weight stopped immediately when perfusion with the fixative was started although there was still some slight weight increase

By light microscopic orientation on semithin sections the pulmonary vascular bed again appeared empty Most of the alveoli, however, were nearly filled with an amorphous substance—most probably representing edema fluid (Fig 2) The outline of the alveoli were in most cases unchanged In some instances, however, the alveoli seemed more or less collapsed In these cases the bulging of the capillaries into the alveoli was quite marked (Fig 2)

By electron microscopy amorphous material was found to be present in most of the alveoli The collagen containing parts of the interstitium appeared expanded by the accumulation of protein rich fluid (Fig 6), and the collagen fibrils were clearly separated

The endothelial cells had the same appearance as such cells from the control experiment preparations No direct evidence of cytoplasmatic swelling or bleb formation was found in the edema preparations The number and size of pinocytotic vesicles also appeared unaltered

Ten intercellular clefts of the endothelium were examined in sections from the control preparations The distance between the stained cell membranes showed a mean value of about 100 Å (range 75—120 Å) Five such cell clefts in sections from the edema preparations were also measured The distance in all these was within 90—110 Å The results of the measurements and the general appearance of the intercellular clefts (Fig 7) indicate that they may have remained unaltered in the edema preparations

The alveolar epithelial cells also appeared to have a structure which was unaltered from that seen in the control experiments

One striking observation in nearly all sections from edema preparations examined (Fig 6 and 7) was that the less electron dense zones of the basement membranes of the endothelium and of the epithelium were much more distinct than in the control preparations These electron lucent zones could be followed from the thinnest parts of the alveolar capillary barrier Fig 7 to the regions of more abundant interstitial tissue (Fig 6

Lungs fixed a[illegible] [illegible]ular [illegible] a[illegible] during net outward fluid filtration from cap[illegible]lar[illegible]

Two experiments were performed in which net outward filtration from the capillaries was induced by increasing the l[illegible] [illegible]al pressure as described in Methods Af[illegible]

4 min the weight was increasing linearly and at a rate of about 0 3 g/min Without the intravascular pressures being altered, the usual procedure for fixation was now followed In all 6—7 min elapsed from the moment when the LAp was increased until the fixation was started The weight of the preparation remained unchanged from the moment the glutaraldehyde perfusion was started and during the whole period of perfusion fixation

Sections from these preparations showed that in some regions of more abundant interstitial tissue there were clear indications of increased interstitial fluid Here the collagen fibrils were separated and there were indications of focal accumulations of fluid (Fig 8) In other interstitial regions however no indications of fluid accumulation were observed Generally the changes in the interstitium although having *the same characteristics, were less pronounced in sections from the filtration experi*ments than in sections from the edema experiments All the alveoli examined appeared to be empty In the regions of these lungs where fluid had accumulated in the larger interstitial regions the electron lucent zones of the basement membranes were definitely more distinct than in sections from the control experiments (Fig 9) When present, such zones were, however less striking than in sections from the edema preparations In regions of the lungs where no fluid accumulation was found the alveolar capillary barrier had the same appearance as in the control experiments The endothelial cell clefts appeared unaffected by the increased intravascular pressure applied in these preparations

In one experiment enough EDTA was added to give a perfusate concentration of EDTA equal to the [Ca_t] At this [*EDTA*] the hydraulic conductivity *of the capil*laries is increased (Nicolaysen 1971 b) When 20 min had elapsed the left atrial pressure was increased by 10 mm Hg followed by fixation 8 min later In this experiment the Krebs Ringer Dextran solution contained 2 5 mM EDTA and the [EDTA] was thus equal to the [Ca_t] The ultrastructure of this preparation (Fig 10 and 11) did not differ from that of the two other preparations fixed during capillary filtration

One pair of lungs which was perfusion fixed *at low left atrial pressure (0 5 mm Hg) but with perfusate concentration of EDTA equal to that of Ca_t* (also [EDTA] = [Ca_t] in the Krebs Ringer Dextran solution) similarly showed a structure which did not differ from that of the control preparations as regards interendothelial cell clefts structure of endothelial cells and appearance of the basement membranes (Fig 12)

Discussion

Perfusion fixation is generally the method preferred to obtain good tissue preservation For ultrastructural studies of changes in capillary permeability and of the ultrastructural effects of transcapillary fluxes of fluid this fixation technique is of special importance The fixative comes into instantaneous contact with the capillary walls and at the same time the intravascular pressures are under full control Thus con

ditions are optimal for arresting the situation which is present immediately prior to start of fixation The few min of perfusion with the Krebs Ringer Dextran solution did not appear to affect the condition of the lung tissue It is also known (G Nicolaysen own observations) that the present lung preparation can be perfused at 28° C with this Krebs Ringer Dextran solution for at least 30 min without any detectable development of edema

No colloid-osmotically active substance was added to the fixative In the control preparations there were no signs of increased amounts of interstitial fluid Neither did the weight of the preparations increase during the perfusion fixation Thus most probably no net flux of fixation solution out of the capillaries took place during the perfusion fixation This observation contrasts to some degree that of Bohman and Maunsbach (1970) who found enlargement of extravascular spaces when different organs (not lungs however) were perfusion fixed with solutions not containing colloid osmotically active substances

An interesting observation as regards this isolated perfused lung preparation may be added Not even in the preparation which had been perfused for the longest period (100 min) before fixation was any sign of damage to cells observed One important factor here obviously is the ventilation of the lungs allowing the oxygen needs of the preparation to be met even when plasma is used as a perfusate The finding of well preserved cells fits with the observation that this preparation can be perfused with plasma at 28° C for more than 4 hrs without spontaneous edema developing (Nicolaysen unpublished)

The alterations in the interstitial tissue of the edematous lungs (EDTA induced) with separation of tissue elements and accumulation of amorphous material correspond to earlier reported alterations during various types of edema formation (Cottrell *et al* 1967 Weibel 1969) The pathways for edema fluid from the interstitium to the alveoli have not been studied in any detail in the present work However no apparent pathway was revealed

During edema development induced by EDTA the capillary permeability to proteins is probably increased No changes in the structure of the endothelial cells or of the interendothelial cell clefts explaining this increase were observed The lack of marked changes in the endothelium could perhaps be expected from the previous finding (Nicolaysen 1971 a) that this EDTA induced increase in permeability is rapidly reversible

Obviously changes in the intercellular clefts could have taken place without being detected in the present study Normally saccharated iron-oxide particles do not pass the intercellular clefts of the endothelium of heart capillaries (Florey 1966) However without detectable structural changes in the intercellular clefts such passage was found after the addition to the perfusate of dini rophenol It has been suggested that a widening of the intercellular clefts of the endothelium and thereby an increased permeability to large molecules takes place also during hemodynamically induced acute pulmonary edema (Pietra *et al* 1969) This proposal was based on the finding that hor[illegible]

thelial cell junctions during perfusion at physiological pressures, whereas the somewhat larger hemoglobin molecule was found to pass in edematous lungs

In order to define the site of leakage in the capillary wall during EDTA induced edema it seems profitable to include in the perfusate molecules which are traceable in the electron microscope, and which do not normally pass this wall Changes explaining the increased hydraulic conductivity of the capillaries induced by lower concentrations of EDTA would probably be more difficult to reveal

The phenomenon of the distinct electron lucent zones of the basement membranes in sections from the edema preparations and in sections from the capillary filtration preparations might be related to the fact that increased movement of fluid out of the capillaries took place during the last period before fixation These zones however, were less distinct and widespread in the experiments of high pressure capillary filtration than in the EDTA edema experiments This discrepancy may be due to the fact that in the edema experiments the rate of weight increase was 70—75 % higher during the last min before fixation than in the filtration experiments Secondly the weight increase, and thereby the total net flux of fluid that had taken place before fixation, was nearly twice as high in the edema experiments as in the filtration experiments This last difference may also explain the observation of less marked alterations in the regions of more abundant interstitial tissue in the high pressure preparations than in the edema preparations

One explanation of the distinct electron lucent zones could be that these represented a fluid layer It is interesting to note then that this change could be observed also in the thinnest parts of the alveolar capillary barrier In these regions larger accumulations of fluid have not been observed in the present work nor in those of several earlier investigators (Weibel 1969) The observations in the experiments here presented might however indicate that fluid can be transferred from the capillaries to the interstitium also in such regions

The same difference in the basement membranes of capillaries of lungs with and without edema seems to be present in the electron micrographs published by Ortega *et al* (1970) However these authors do not comment on this point At an EDTA concentration of 50 mM Clementi and Palade (1969) found that the endothelial cells became detached from the basement membrane in the intestinal capillaries In their study this separation could have been caused by an edema formation On the other hand it might be a direct effect of the extremely low concentrations of Ca (and/or Mg^{++}) resulting from the high EDTA concentration used The distinct laminae rarae of the basement membranes observed in the present study during edema development was most probably not caused by the low [Ca] and [Mg] per se, as this phenomenon was seen also during net capillary filtration at normal [Ca] and [Mg^{++}]

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Key to abbreviations used in figures

A	Alveolus	I	Interstitium
AD	Alveolar duct	IC	Intercellular cleft of endothelium
AE	Alveolar edema	INC	Interstitial cell
AF	Accumulated fluid	M	Mitochondrium
BM	Basement membrane	MT	Microtubulus
C	Capillary	N	Nucleus
CF	Collagen fibrils	NE	Nuclear envelope
EL	Elastic tissue	PV	Pinocytotic vesicle
EN	Capillary endothelium	R	Ribosomes
EP	Alveolar epithelium	TM	Tubular myelin figures
ER	Endoplasmatic reticulum	V	Small vein
EZ	Electron lucent zone of basement membrane		

Fig 1 Semithin section from a control specimen Both the alveoli (A), the capillaries (C) and a vein (V) appear empty The alveoli seem well inflated Toluidine blue ×385

Fig 3 Control specimen representing a thicker part of the alveolar capillary barrier Between the basement membranes (BM) of the endo and epithelium (EN and EP, respectively) an extension of an interstitial cell (INC) is seen and in another place tissue representing collagen fibrils and elastic tissue (EL) The nucleus (N) of an endothelial cell is seen at the upper right The cytoplasma of this cell contains a mitochondrium (M), granular endoplasmatic reticulum (ER) and free ribosomes (R)

Fig 4 Control specimen representing the alveolar capillary barrier at a site of minimal barrier thickness The basement membranes of the endo- and epithelium appear fused and these membranes seem nearly homogenous electron dense Note the densely arranged pinocytotic vesicles (PV) in the endothelial cell The epithelial cell contains fewer vesicles and the cytoplasma is more electron dense than the cytoplasma of the endothelial cell

Fig 5 Control specimen showing an intercellular cleft (IC) of the endothelium in a region where the alveolar capillary barrier is at its thinnest Part of a microtubulus (MT) can be observed in the cytoplasma of the endothelial cell

Fig 6 Edema specimen showing focal accumulations (AF) in the interstitium of amorphous material representing edema fluid Collagen fibrils seem to be separated from each other One alveolus appears nearly filled with an amorphous material the other one shows only traces of such material

Fig 7 Edema specimen with region of fused basement membranes The junction between two endothelial cells have the same appearance as those observed in sections from the control preparations Over large areas distinct electron lucent zones (EZ) of the basement membranes are clearly seen

Fig 8 Section from preparation fixed during high pressure capillary filtration at normal intravascular $[Ca^{++}]$ and $[Mg^{++}]$ The interstitial space (I) appears widened Note the clearly visible electron lucent zones of the basement membranes

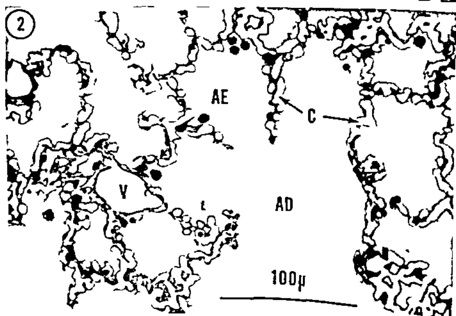
Fig 9 Same type of preparation as in fig 8 Section including region of thinnest alveolar capillary barrier As in Fig 8 the electron lucent zones of the basement membranes are more distinct than in the sections from control specimens Note the presence in the alveolus of myelin figures (TM) probably representing surfactant

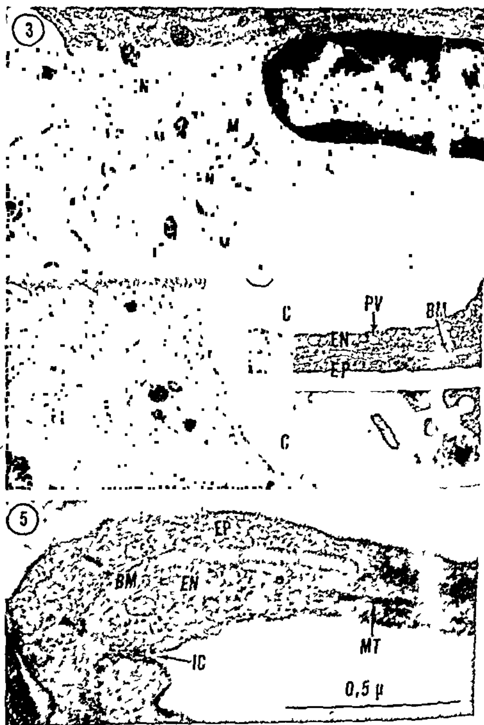
Fig 10 Section from preparation fixed during high pressure capillary filtration About 25 min before start of fixation EDTA had been added to the plasma so as to give $[EDTA] = [Ca_t]$ Again the electron lucent zones of the basement membranes appears more distinct than in the control specimens

Fig 11 Section from the same preparation as Fig 10 showing air blood barrier of minimal thickness Note the endothelial pinocytotic vesicles which is nearly extending from the luminal side to the basal plasma membrane of the endothelial cell In some places the cytoplasma of the epithelium is extremely scarce

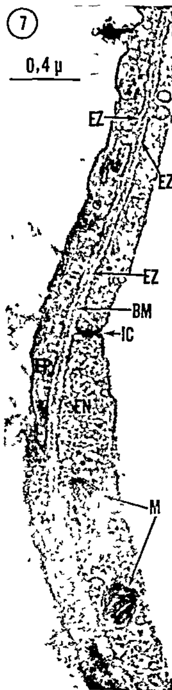
Fig 12 Preparation fixed at normal intravascular pressures EDTA had been added about 25 min before start of the fixation so as to give $[EDTA]$ $[Ca_t]$ The ultrastructural appearance of the alveolar capillary barrier is indistinguishable from that in comparable sections from the control preparations

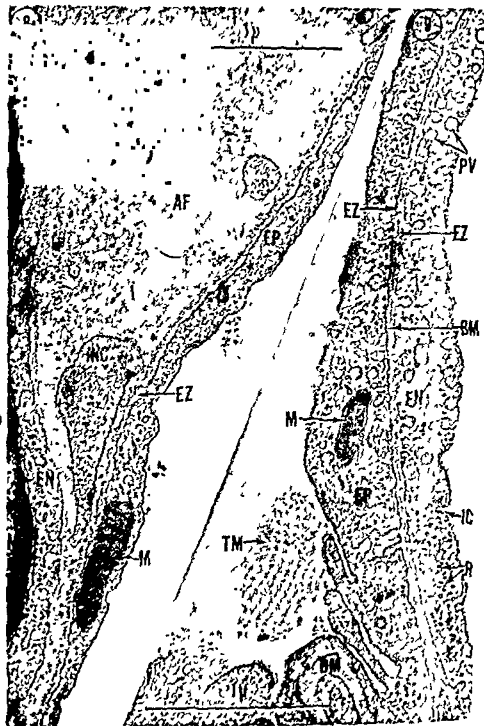
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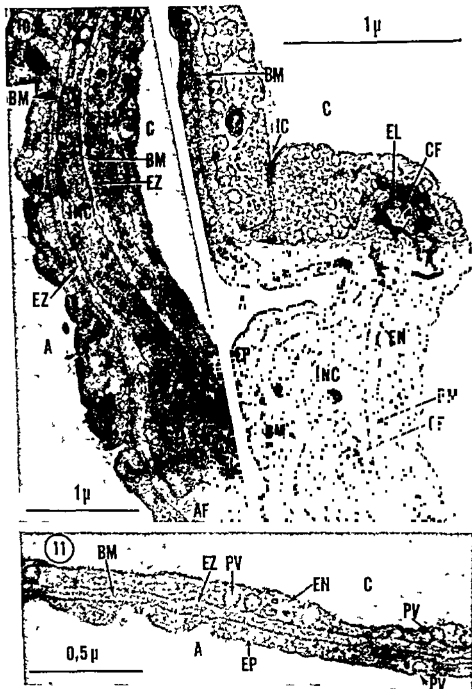



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Abstracts from Meeting of the Scandinavian Physiological Society in Bergen 7—8 May 1971

DEMONSTRATIONS

D 1

Sleep in the Cat A Parallel Increase of Deep Slow Wave Sleep and REM Sleep Following Total Sleep Deprivation

By R. Ursin *Institute of Physiology, University of Bergen, Norway*

Sleep in cats may be divided into three stages Light slow wave sleep (LSWS), deep slow wave sleep (DSWS) and rapid eye movement sleep (REM sleep) Earlier studies on naturally sleeping cats have suggested a functional dissociation between LSWS and DSWS and a particular relationship between DSWS and REM sleep (Ursin 1968, 1970) In the present study, sleep deprivation was used as a tool to study these relations further According to findings in man (Berger and Oswald 1962) one would expect an increase in DSWS in postdeprivation sleep According to the earlier findings one would expect that an increase of DSWS would be followed by an increase of REM sleep and a decrease of LSWS

In 10 cats, sleep was recorded for 24 h after 12 h and 24 h total sleep deprivation and after no sleep deprivation The total quantities of DSWS and REM sleep in the 24 h recordings increased with deprivation, as did the relative proportion (per cent of total sleep) of these sleep stages The total quantity of LSWS did not change with sleep deprivation but LSWS per cent of total sleep decreased The changes were most pronounced after 24 h deprivation and in the first hours of recovery sleep

Sleep deprivation increased the number of sleep cycles, reduced LSWS episode length and tended to increase DSWS and REM sleep episode length The length of the sleep cycle was not altered by sleep deprivation

The results support the earlier findings of a functional dissociation between LSWS and DSWS and of a particular relationship between DSWS and REM sleep

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D 2

Electrical Stimulation of the Brain and Learning in the Cat

By H SUNDBERG *Institute of Physiology, University of Bergen, Norway*

During electrical stimulation of the brain the cat often turns its head towards the side contralateral to the stimulated hemisphere, pricks its ears and opens its eyelids Stimulation of a variety of brain sites gives this reaction amygdala the caudate nucleus, cortex thalamus and the mesencephalic reticular formation Descriptively this response is identical to the orienting reaction shown by the cat to external stimuli, such as a tone The problem raised in this research is to determine whether the orienting response elicited by brain stimulation really is identical to the species specific orienting reaction of the cat when it is experiencing something novel in the surroundings Previous studies have shown that the response to brain stimulation may habituate, as does the orienting reaction to external stimuli (Ursin *et al* 1967, 1969 Wester 1971) The present research demonstrates that the response to brain stimulation is identical to the orienting reaction to external stimuli in a third way When the stimulus is given signal value in a learning situation, the orienting response gradually disappears and is replaced by the learned behavior adequate for the situation.

Electrodes were implanted in the caudate nucleus in cats Electrical stimulation of the caudate nucleus elicited the orienting response All points in the caudate showed the habituation phenomenon Following habituation the brain stimulation was used as a conditioned stimulus Five seconds of brain stimulation was given before electrical shock was delivered to the floor of a chamber containing a safe platform Acquisition of the avoidance response using brain stimulation as the signal occured in the same fashion as when a tone signal was used the orienting reaction at signal onset occurs during the first learning trials but gradually disappears and is replaced by rapid avoidance responding with further trials

These experiments support the h[illegible]
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D 3

Extrapleural Measurements of Blood Flow in the Arch of the Aorta with Electromagnetic Flowmeter

By A. Kirkebö, *Institute of Physiology, University of Bergen, Norway*

Standard methods for application of measuring devices directly on the arch of the aorta requires thoracotomy and the use of a respirator or a long recovery time after chronic implantation of probes.

In order to measure blood flow in hedgehogs and in rats, an electromagnetic flowprobe can be introduced from the neck along trachea through the interclavicular fascia to the aortic arch. The aorta can be dissected from its supporting membranes by a blunt instrument without rupture of the mediastinal pleura. No respirator is needed even when the wound is still open. Only skin and fascia are cut and the probe lead can be well fixed under the clavicula before the wound is closed. Thus this method also permits direct measurements of aortic blood flow a short time after recovery from anesthesia.

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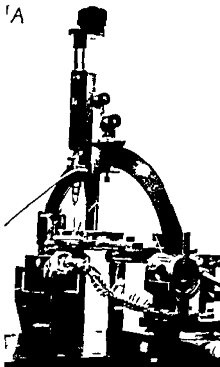
D 4

A Closed Chamber for Microelectrode Recording from the Brain

By S. A. Andersson and Y. Kallstrom, *Department of Physiology, University of Göteborg, Sweden*

Intracellular microelectrode studies in the brain are technically difficult because of movements from the heart beats and the respiration. In order to minimize these difficulties a closed chamber system has been developed which also allows recordings from the brain of the cat according to Horsley Clarke coordinates (Fig. 1 A).

Initially the animal is fixed with earplugs in a Horsley Clarke headholder (Fig. 1 B). The bone of the skull is opened over the desired area and a metal cylinder, 15 mm in diameter, is attached to the bone by cement and metal screws. Medio-lateral and anterio-posterior coordinate reference points are indicated on the exposed tissue. The animal is then removed from the Horsley Clarke stand and the cylinder then acts as a headho'der and is fixed into a metal disc with a corresponding opening (Fig. 1 C). The upper surface of the disc has a circular grove with a rubber ring against which a transparent plastic disc can be pressed, thus closing the chamber which is filled with mineral oil. Static pressure on the brain is eliminated by a thin canula through the plastic disc and a polyethylene tubing while transient pulsations are suppressed. The microelectrode driver is centered to pass the electrode through a Teflon fitting in the disc. The disc can slide over the rubber ›





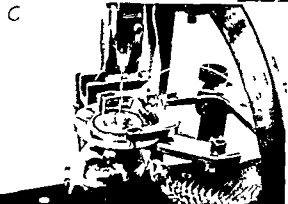


Fig 1 Photographs of the micromanipulator with the closed chamber arrangements

coordinate table allows penetrations into subcortical structures in relation to the indicated reference points

With this technique satisfactory conditions for intracellular studies were obtained without pneumothorax and artificial respiration or any other precautions Intracellular recordings have been possible both from interneurones and relay cells in the thalamus, frequently cells with a membrane potential of at least 50 mV were kept for 30 min or more

D 5

Stimulation and Recording with Closely Spaced Microelectrodes

By E Eide *Department of Physiology, University of Göteborg Sweden*

Extracellular stimulation of neurones with microelectrodes requires high voltage pulses and potentials recorded with another microelectrode in the vicinity are often severely distorted Due to stray coupling capacitance (C in Fig 1) voltage transients in the stimulating electrode give a current flow in the recording electrode This causes a voltage drop in the recording electrode resistance R_p — the stimulus artefact. If it exceeds the voltage handling capability of the amplifier the stimulus artefact will be prolonged due to capacitive charge Even if the stimulus pulse itself

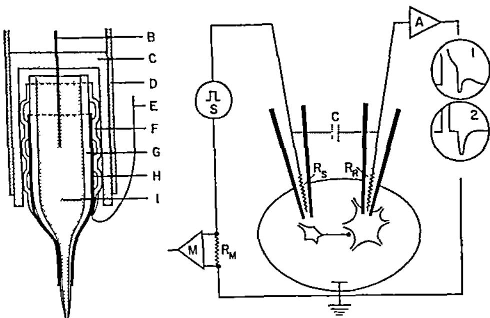


Fig 1 *Left* Microelectrode (G) painted with conducting paint (H) (Degussa Leitsilber 200) A shrinkable tubing (F) (Raychem RNF 100) heated to constrict about every 5 mm provides insulation and fixation The unheated tube segments fit in the insulating electrode holder (C) The shield (D) surrounding the holder overlaps the electrode shield Electrolyte (I) connected via chlorided silver wire (B) to amplifier or stimulator (E) connecting wire from shield

Right Schematic drawing of experimental situation Stray coupling capacitance C is reduced by shielding one or both of the electrodes Stimulator (S) provides voltage pulses, the actual current being monitored at R_m (100 Ω) by floating amplifier (M) 1) Response to stimulation of interneurone, recorded intracellularly from motoneurone a few mm away, time between end of stimulus and start of response being about 200 μs Stimulation and recording without shielded electrodes 2) same as 1), but stimulating electrode shielded

has ended before the onset of the response, this prolongation might severely interfere with the recording of the response A reduction of the coupling between the electrodes is then necessary

This can be accomplished by shielding either electrode with electrically conducting paint (see Fig 1) For stimulation the shield should be grounded For recording the shield can be electrically driven from the amplifier to reduce input capacitance The painted part of the electrode must not be inserted into the tissue For this reason the electrode shield should end about 1 mm above the maximal insertion level If needed a shield for the remaining coupling capacitance between the unshielded parts of the electrodes can be provided by a piece of filter paper wetted in saline and placed between electrodes in contact with the specimen

D 6

Gastric Acid Response to Distension of the Stomach in Man

By S Bergegårdh and L Olbe Department of Surgery II Sahlgrenska sjukhuset, Goteborg, Sweden

Animal experiments have shown that the gastric phase of gastric acid secretion involves two main stimuli cholinergic activation of the acid secreting glands and release of gastrin from the antrum The cholinergic activation is produced by distension of the acid secreting part of the stomach (corpus fundus) and is elicited via intramural and vago vagal reflexes Release of gastrin is produced by distension of the antrum or by certain chemical agents under the prerequisite of a non acid antral milieu (a low antral pH inhibits gastrin release) In the dog distension of corpus-fundus or antrum both results in a marked acid secretion

In the present study the acid response to separate distensions of corpus fundus and antrum have been investigated in man (normals and ulcer patients) The distension was produced by a balloon taped on the nasogastric tube used for collection of the gastric juice The correct position of the balloon was intermittently checked on an X ray television screen During antrum distension a neutral buffer solution together with an inert marker was continuously infused into the stomach (1 lit/hour) to keep the gastric content at pH 6—7 despite acid secretion By the titration of the gastric content and unmixed buffer to pH 9 5 the acid secretion can be calculated Distension of the antrum with 50 150 and 200 ml did not increase basal acid secretion in 5 patients Nor did the antral distension increase the basal acid secretion in 7 expts in which the gastric content was kept neutral by buffer infusion. Distension of the corpus-fundus on the other hand resulted in a successively increasing acid secretion with increasing balloon volume In 10 patients acid secretion reached maximum with a balloon volume of 600 ml The maximal acid response to corpus-fundus distension was 52 % (range 34 %—83 %) of the maximal acid response to pentapeptide gastrin. Combination of submaximal stimulation by corpus-fundus distension (balloon volume 150 ml) and an i v infusion of pentapeptide gastrin in a threshold dose (4 expts resulted in an additive secretory effect but no potentiation

Conclusion

Distension of the human stomach results in a marked acid secretion This secretion is mainly due to distension of the corpus-fundus (probably a cholinergic reflex activation) because antral distension does not produce enough gastrin release to elicit acid secretion and gastrin does not potentiate the acid response to corpus-fundus distension.

D 7

Developmental Defects Produced by Brain 5-hydroxytryptamine Depletion in Rat Pups

By K. HOLE *Institute of Physiology University of Bergen Norway*

In the human disease phenylketonuria (PKU) there is not only a defect in hydroxylation of phenylalanine secondary to this there is a defect in 5 hydroxyindole metabolism yielding a low brain 5 hydroxytryptamine (5 HT) concentration It has been proposed that the low brain 5 HT may be a factor of particular importance for the brain damage in this disease (Woolley and van der Hoeven 1965) p-chlorophenyl alanine (pCPA) gives a rather selective depletion of brain 5 HT and may therefore be useful for testing this hypothesis

Brain 5 HT was depleted to 20 % of control values in 71 rat pups during the first 7 weeks of life by intraperitoneal injections of pCPA Serum phenylalanine was only moderately increased by the treatment most of the time the serum concentrations were less than about 10—12 mg/100 ml and Phenistix tests for phenylpyruvic acid in the urine were all negative Serum tyrosine concentrations were normal The brain weight was significantly reduced evident after 2 weeks of treatment and also at the end of the experiment 52 days after the last injection

The decrease in brain growth was accompanied by behavioral changes evident later in life The behavioral tests were spontaneous alternation activity test open field habituation to auditory stimulation Lashley III maze and passive avoidance The first test was given 4 weeks after the last pCPA injection The changes observed were moderate but consistent in all the tests There were no learning deficits or clear motivational changes However in all the tests there was evidence of a reduced arousal level in the pCPA treated rats latencies were long exploration was low habituation to new environment was fast and the reactivity was low In a previous experiment a similar defective arousal function was found in rats fed L-phenyl alanine (PA) in this model for PKU there is also a low brain 5 HT In human PKU a similar behavioral deficit is also observed (Anderson *et al* 1969) The importance of the 5 HT depletion in reducing the brain growth both in pCPA and PA treated rats was confirmed in another experiment demonstrating that additional injections of 5 hydroxytryptophan restoring brain 5 HT in both preparations have a protective effect against the retardation in brain growth otherwise produced

A low brain 5 HT seems to be an important factor in producing the faulty brain development both in pCPA treated and PA treated rat pups possibly it may be so even in human PKU Drugs depleting brain 5 HT may have similar deleterious effects in humans and should not be used in children in the first 2—3 years of life or during pregnancy

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D 8

Effect of Strenuous Arm and Leg Training on Pulmonary Ventilation, Metabolism and Blood pH During Submaximal Exercise

By J P CLAUSEN K. KLAUSEN B RASMUSSEN and J TRAP JENSEN *Laboratory for the Theory of Gymnastics, University of Copenhagen & Department of Clinical Physiology, Bispebjerg Hospital, Copenhagen, Denmark*

10 young male subjects trained daily for 5 weeks on a bicycle ergometer The work load was selected to give the subjects a heart rate exceeding 170 beats/min in each training session 5 of the subjects trained with their arms and 5 subjects trained with their legs The following parameters were measured before and after the 5 weeks training period at rest and during two submaximal work loads Oxygen uptake ($\dot{V}_{O_2}$), pulmonary ventilation (V_E), arterial lactate and pH and venous lactate and pH from the working muscles Samples for lactate and pH were taken at intervals during each test work The present data includes only the 5 min blood values

Arm training The V_{O_2} during arm work was decreased with apr 100 ml/min at both work loads while no change was found during leg work The respiratory exchange ratio (R) was decreased by 0 12 during arm work No change of R was seen during leg work. V_E was decreased by 4 0 and 9 0 l/min at the two work loads in arm work while no change was seen in leg work. Arterial and venous lactate in arm work was decreased by 10—21 mg % while the decrease in leg work as only 1—4 mg % No change in arterial pH was seen during arm work while the venous pH showed a marked increase (over 0 02) In leg work a decrease of arterial and venous pH was found at the low work load (0 017) while at the higher work load both values were increased (0 007—0 025)

Leg training In leg work a decrease of V_{O_2} of 100 ml/min was found at the highest work load while no change was found at the low work load No change of V_{O_2} was seen in arm work. R was decreased by 0 06 in leg work and only by 0 01 and 0 03 in arm work V_E was decreased by 5 5 and 9 0 l/min respectively in leg work while no change was seen in arm work. The arterial and venous blood lactate in leg work was decreased by 12 and 23 mg % at the low and high work load respectively No change of the lactate concentrations was seen in arm work The pH (both arterial and venous) was increased in leg work (0 008—0 024) Almost no change of pH was seen in arm work

The results suggest a predominant peripheral training effect of leg training and arm training respectively since practically no changes were found in arm work after leg training and vice versa.

D 9

The Hemodynamic Consequences of Regional Hypotension in Spontaneously Hypertensive and Normotensive Rats

By B. Folkow, M. Gurévich, M. Hallbäck, Y. Lundgren and L. Weiss
Department of Physiology, University of Göteborg, Sweden

In 3 week old, spontaneously hypertensive rats (SHR) and normotensive control rats (NCR) the aorta was ligated distally to the renal arteries 6—16 weeks later these rats had a 30—40 % lower blood pressure in the hindquarters than ordinary NCR In 20 paired experiments the hindquarters of one ordinary NCR and one aorta-ligated NCR or SHR were perfused at constant flow with an oxygenated plasma substitute In the aorta-ligated NCR and SHR, resistance at maximal dilatation was reduced 40—50 % and they displayed a decreased steepness of the dose response curve and a decreased maximal pressor response, but unchanged threshold to graded noradrenaline doses, as compared to unligated shamoperated NCR

The results indicate the presence of a structural change of the resistance vessels in the artificially hypotensive hindquarters of NCR or SHR, with a regression of the media mass and a widending of the lumen at maximal dilation, largely in proportion to the lowered pressure level When compared to unligated SHR these hemodynamic differences were still larger, giving different resistance curves for NCR, SHR and the aorta ligated NCR-SHR, respectively, each related to the regional blood pressure existing before the perfusion Thus, a regionally decreased

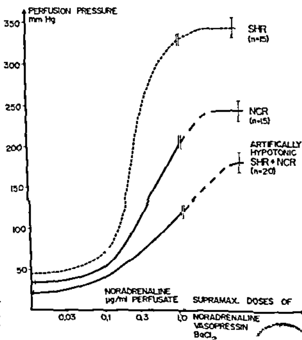


Fig 1 Average "resistance curves for 15 SHR 15 NCR and 20 hypotonic hindquarters (for details see Folkow *et al* Acta physiol scand 1970 *80* 93—106)

blood pressure in an otherwise hypertensive animal seems to prevent the development of an adaptive increase of the media in the corresponding resistance vessels despite their probable exposure to the same neuro-hormonal excitatory impact as in other regions stressing the basically *local* nature of this structural change In general an altered pressure load decreased or increased appears to affect the design of the resistance vessels largely accounting for the different levels of hemodynamic equilibrium in hypotension and hypertension as compared to normotension

D 10

Lactate/Pyruvate Ratio in Muscular Work

By C Olsen and E Strange Petersen *Institute of Physiology University of Aarhus Denmark*

Keul *et al* (1968) measured the lactate/pyruvate ratio (L/P ratio) across working muscles in man and found it significantly higher on the arterial than on the venous side A possible explanation of this finding is that resting muscles having a diminished flow during work with other groups of muscle might contribute with a high L/P ratio and thus increase the ratio in the arterial blood The present study was designed to test this possibility

5 healthy subjects exercised on a bicycle ergometer for 10 min at a load giving a steady state heart rate of 180 Samples of arterialized capillary blood and venous blood were taken from the ear lobe and from the axillary vein respectively at rest and repeatedly during work and recovery Lactate and pyruvate were determined in all samples with an enzymatic method

At rest venous lactate concentration was higher than arterial (Table I) Throughout the work period however *arterial concentrations were higher* than venous indicating that lactate from resting muscle does not contribute to the high concentration in arterial blood Maximal concentrations of lactate coincided with the termination of work Pyruvate concentrations were higher in arterial than in venous blood at rest and throughout the work period A steep rise in pyruvate concentrations during the first 5 min of recovery was seen in all subjects L/P ratio increased earlier and steeper in arterial than in venous blood and maximal values were usually seen

Table I Mean values for lactate pyruvate and L/P ratio in arterial and venous blood at rest and mean maximal values during exercise Standard errors of the means in brackets ($n = 5$)

	Rest		Exercise	
	arterial	venous	arterial	venous
Lactate mM	0 50 (0 05)	0 65 (0 07)	7 7 (1)	6 5 (1)
Pyruvate mM	0 0F9 (0 016)	0 065 (0 001)	0 380 (0 067)	0 320 (0 067)
L/P ratio	8 (1	10 (1)	32 (3)	29 (3)
Heart rate	75 7		178 (5)	

before the exercise period had ended, being almost identical in arterial and in venous blood

In conclusion, the study has shown a significant uptake of both lactate and pyruvate by the resting muscle during the exercise period without any significant change of the L/P ratio The finding of higher L/P ratios in arterial than in venous blood from working muscles by Keul *et al* (1968) can therefore hardly be explained by a high ratio contribution from resting muscle

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D 11

Renal Function in the Hibernating Hedgehog

By G CLAUSEN and A STORESUND *Institute of Physiology, University of Bergen, Norway*

The present study was made to decide whether the urine found in the bladder of hedgehogs hibernating at about 5° C is produced at this body temperature We therefore determined the concentrations of the Na, Cl, and urea in renal tissue and the osmolarity of tubular fluid at different levels between the renal cortex and the tip of the papilla and in plasma and urine The lack of increasing concentrations of Na Cl and urea from cortex to papilla show that the renal counter current multiplier system is practically inoperative during hibernation

The osmolarity of papillary collecting duct fluid was similar to that of plasma However the bladder urine contained 1100 mOsm/l It cannot, therefore have been produced during hibernation, but must have been left in the bladder from the previous arousal period

C 1

Osmotic and Ionic Conditions in Pelagic Teleost Eggs

By H Leivestad *Zoological Laboratory, University of Bergen, Norway*

The teleost egg can be considered as consisting of two separate liquid compartments, a) The egg proper enveloped in the perivitellin membrane and containing the proto plasm and the yolk, b) The perivitellin space, limited by the chorion a structureless liquid wherein the egg proper floats freely Previous data seemed to suggest that the chorion is permeable to water and salts, whereas the egg proper retains the low osmolarity of the ovarian fluid and thereby acquires its ability to float in sea water The teleost egg, in contrast to most invertebrate eggs, does not swell or shrink in response to ionic changes of the environment

Working mainly with cod eggs at different stages of development, the present author has determined the ionic composition, the freezing point depression and colloid osmotic relationships in experiments with varying strength of the different ionic species of the environment

It can be concluded that the perivitellin fluid is in ionic equilibrium with the surrounding medium. but containing some colloid, giving rise to the turgor of the egg and the distension of the chorion In the egg proper the ionic ratios are essentially intra-cellular (high K^+ low Na^+ and Cl^-), and the sum of the common ions is extremely low (80 mM) compared to sea water (1000 mM) The freezing point depression of the egg is 0 6° C to 0 8° C (sea water 1 8° C) That these characteristics have not been achieved by rendering the perivitellin membrane completely inpermeable is shown by the fact that water is readily withdrawn from the egg by an external solution containing colloids The egg also loses water in response to dinitrophenol

C 2

Effects of Increased Work Load in Vitro on Heart Protein Synthesis

By A. Hjalmarson and O Isaksson *From the Department of Physiology, University of Göteborg Sweden*

Hypertrophy of the heart is important for development of general compensation when the heart is exposed to increased work load From *in vivo* studies of ^{14}C-amino acid incorporation into heart protein it has been suggested that the increased protein content of the hypertrophied heart is due to acceleration of protein synthesis (e g Meerson 1969) The mechanical events triggering these biochemical events are poorly understood at present The protein synthesis may be regulated by 1) transport of free amino acids into the heart cells 2) activation of intracellular amino acids, or 3) polymerization of activated amino acids into protein on the ribosomes

In the present study the effect of well defined work loads on protein synthesis in the isolated, working rat heart was investigated (Hjalmarson *et al* 1969) Hearts from normal rats were perfused with cannulated left atrium and aorta for 120 min with Krebs bicarbonate buffer containing all amino acids in normal plasma concentrations with glucose or palmitate as substrate The rate of heart protein synthesis corporation rate of ^{3}H phenylalanine from 291 ± 21 to 414 ± 27 dpm/100 μg protein fraction of the heart. Sucrose gradient analysis of an 8000 g supernatant of heart homogenate was performed to study effects of work load on ribosomal aggregation An elevation of the aortic diastolic pressure from 40 to 100 mm Hg increased in corporation rate of ^{3}H phenylalanine from 291 ± 21 to 414 ± 27 DPM/100 μg protein. Ribosome profiles of hearts perfused at the higher work load showed increased number of heavier polysomes and less large and small subunits (60 s and 40 s) Thus, increased pressure work load stimulated aggregation of ribosomes into polysomes and facilitated initiation of peptide chains On the other hand increasing left atrial filling pressure (preload) at a constant perfusion pressure (afterload) did not change the incorporation rate of ^{3}H phenylalanine, although the cardiac output was increased 3 times Both end systolic and end diastolic volumes were increased at higher afterload, while higher preload increased end diastolic but not end systolic volume

It is thus clear that increased work load *in vitro* can increase the protein synthesis and that aggregation of ribosomes into polysomes with facilitated initiation of peptide chains might be important for this effect The increased end systolic volume with sustained stretch of myocardial fibers due to elevation of pressure work load is suggested to mediate this effect

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C 3

Increased Mechanical Efficiency in Contracting Skeletal Muscle induced by Isoproterenol

By S AKRE and O D MJOS *Institute for Experimental Medical Research, Ullevål Hospital University of Oslo, Norway*

The effect of isoproterenol on generated force and energy cost of the contracting skeletal muscle has been studied in an *in situ* preparation of gracilis muscle from pentobarbital anesthetized dogs The distal tendon of the muscle was cut and tied to the free end of a rigid steel rod Contraction force could be measured from the recorded deflection of the rod The contractions were virtually isometric The energy expended during muscle contraction was calculated from the heat generated in the muscle during contraction When heat loss from the muscle was prevented, energy expended was accumulated as heat in the muscle. Temperature rise measured by

1—3 thermocouples sewn into the muscle could therefore be used to quantitate the energy cost of the contractions (Akre and Aukland 1970) The muscle was con tracted by stimulating the motor nerve with monophasic pulses (3 msec 6—8 V) from a Grass stimulator at frequencies of 5 10 20 and 30/sec

Mean force increased with increasing frequencies reaching a maximum of about 3 kg at a rate of 30/sec For a given stimulation frequency the contraction force during isoproterenol infusion (2—3 μg/min i v) was essentially the same as in the control period The muscle temperature increased linearly throughout the contrac tion period indicating constant heat accumulation rate For a given mean force the energy turnover was always higher before than during infusion of isoproterenol. At a stimulation frequency of 30 pulses/sec the temperature rise in the presence of isoproterenol corresponded to a mean metabolic rate of 0 75 cal/min g compared to 1 11 cal/min g in control state It is concluded that the —
gracilis muscle calculated ^
considerably increased b

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C 4

hibition of Palatal and Laryngeal Reflexes by Afferent Stimulation

By C Lindqvist and A Mårtensson *Department of Physiology Karolinska Institutet Stockholm Sweden*

There is close interaction between the muscles of the palate the upper pharynx and the larynx as regards functions such as swallowing and phonation The intrinsic laryngeal reflexes and reflex connections from lingual afferents to laryngeal muscles have previously been studied (e g Mårtensson 1963 Lindquist and Mårtensson 1969) Further experiments have now been undertaken to study the role played by the lingual and certain pharyngeal and pulmonary afferents in the control of reflex excitability of palatal and laryngeal muscles Dogs under chloralose anesthesia were used as experimental animals

Single stimuli were applied to the internal laryngeal or glossopharyngeal nerve, these resulted in reflex discharges of a duration of about 10 msec and a latency of 10—12 msec in mm pterygopharyngeus and levator veli palatini and in some experi ments in m tensor veli palatini Occasionally inspiratory spontaneous activity was recorded in these muscles The reflex responses were however less pronounced during inspiration and insufflation hence pulmonary stretch receptors seem to exert a depressing influence on these reflexes

In another series of experiments the reflex excitability in the adductors of the larynx and the palatal muscles was tested following application of a conditioning

afferent stimulus The reflexes were elicited by electrical stimulation of the internal laryngeal or glossopharyngeal nerve Single subliminal conditioning stimuli were applied to one of the following nerves, *viz* the internal laryngeal, the glossopharyngeal, the lingual or the hypoglossal nerve As a rule, the conditioning stimulus was followed by a period of reduced excitability Regardless of the nerve thus stimulated the inhibitory period was well above 100 msec, the depression might often be maintained for several hundred msec Thus, *e g* a reflex set up in the m levator veli palatini by stimulation of either the glossopharyngeal or the internal laryngeal nerve after a conditioning stimulus applied to the lingual nerve was depressed for 400 msec. This long lasting inhibition suggests a presynaptic inhibitory mechanism This possibility was tested by studying the effect of picrotoxin A reflex evoked in the thyroarytenoid muscle by stimulation of the internal laryngeal nerve was strongly depressed by a conditioning stimulus applied 100 msec in advance to the lingual nerve After i v injection of picrotoxin the inhibition was abolished but was obtained once more when the effect of the drug had worn off This speaks in favor of the proposed inhibitory mechanism In this case lingual afferents exerted a control of reflex effects of impulses in laryngeal afferents through presynaptic inhibition, but the reverse may also be the case since a trigeminal dorsal root reflex (TDRR) could be elicited in the lingual nerve on stimulation of the internal laryngeal nerve, it is thus evident that there is an interneuron chain connecting laryngeal afferents with the primary afferents in the lingual nerve

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C 5

Time Difference between Onset of Spindle Acceleration and Skeletomotor Activation in Isometric Voluntary Contractions in Man

By Å B VALLBO *Department of Physiology, Biological Institute, University of Umeå, Sweden*

In many types of motor acts studied on anesthetized and decerebrate preparations it has been shown that the fusimotor and the skeletomotor systems are not activated simultaneously, at the onset of a contraction the fusimotor outflow usually precedes the onset of the skeletomotor activity This suggests that an essential function of the fusimotor system is to adjust the excitability of the skeletomotor neuron pool before the skeletomotor outflow is triggered from supraspinal structures It is also consistent with the notion that skeletomotor contractions are totally controlled by spindle afferent discharge

It has earlier been shown that the afferent discharges from muscle spindles increase when the surrounding skeletomotor fibres contract under isometric conditions during voluntary contractions in man (Vallbo 1970 a 1970 b)

In the present study the time of onset of spindle acceleration was assessed in relation to the onset of skeletomotor contraction under these conditions Impulses from single spindle afferents from the wrist and finger flexor muscles were recorded as well as the electromyographic activity of the muscle portion in which the spindle was located The contractions were varied over wide limits with regard to the rate of the rising phase

It was found that spindle acceleration virtually always started *after* the onset of the skeletomotor contraction The time shift between the two varied considerably from one test contraction to the other However, the time shift was often 10—50 msec, suggesting that there was a simultaneous onset of fusimotor and skeletomotor outflow from the spinal cord

The findings indicate that voluntary contractions are initiated directly by descending impulses impinging upon the segmental organization and not by reflex effects of spindle afferents

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C 6

Plasma Corticosterone Levels during Active Avoidance Learning

By G D COOVER S LEVINE and H URSIN *Institute of Physiology, University of Bergen Bergen Norway and Department of Psychiatry Stanford University Medical School U.S.A*

Plasma corticosterone level is a useful indicator of the level of activation following a stress like electrical shocks In avoidance learning the individual is able to avoid the shock as by running to a safe position or pressing a lever The ' two factor theory of avoidance (MOWTER 1960) postulates that the avoidance act is fear reducing Animals performing perfectly in an avoidance situation will show clear reduction in their fear Alternative theories require that fear must remain if perfect performance is to be maintained If the two-factor theory is correct the activation level and plasma corticosterone level should decrease when perfect performance is reached

Seventeen male Moll Wistar rats (250—300 g) were given extended training in a standardized two way active avoidance apparatus An auditory stimulus (white noise) of 5 sec duration preceeded electrical shock (0 5 mA) If the rat crossed to the other side of the box over a 5 cm hurdle within the first 5 sec of the sound shock was avoided Intertrial interval was 1 min 20 sessions of 10 trials per day were given Plasma corticosterone levels were determined following 4 of the sessions Fluorometric determination of corticosterone (Glick *et al* 1964) was made on blood samples drawn from the jugular vein under ether anesthesia 20 min after the beginning of the particular avoidance session

The majority of the subjects attained near perfect shock avoidance performance within 5 to 10 sessions The plasma corticosterone level was lower on Day 6 than on Day 1 During the 10 to 15 practice sessions that followed, they maintained near perfect avoidance performance, and the plasma corticosterone level declined even further Defecation (boluses) exhibited a similar decline

The findings support the two-factor theory of avoidance A rat that lives in a potentially dangerous situation does not show high activation levels (is not "stressed") when it has learned to cope with the situation

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C 7

Initial Ventilatory Response during Muscular Exercise in Man

By J INGEMANN JENSEN, E STRANGE PETERSEN and H VEJBY-CHRISTENSEN
Institute of Physiology, University of Aarhus, Denmark

The general validity of the concept, that the ventilatory response to muscular exercise can be separated into a rapid neural response and a slower humoral response (Dejours and Teillac 1963) has recently been questioned (Beaver and Wasserman 1968) Initial ventilatory changes during work are likely to be influenced by the experimental situation as such, by the way the starting-order is given, by conditioning (*cf* Torelli and Brandi 1961), by the subjects' knowledge of the purpose of the study, and by the enthusiasm of the investigator

5 subjects aged 21—24 entered the present study, 4 of 5 having no knowledge of the purpose of the study They sat on a bicycle ergometer (Elema Schonander) modified to keep the flywheel running during intervals between work in order to ensure constant load from the beginning, 500—1000 kpm/min (heart rate 130—150) Ventilation was measured breath by-breath with a spirometer on the expiratory side of an open circuit respiratory system In an attempt to standardize the experimental procedure, tapes with previously recorded instructions and protocols were employed 5 different protocols were used on 5 different days Each protocol lasted 45 min and consisted of a total of 8 orders separated by intervals of 3—6 min Both the sequence of the orders and the time interval between orders were randomized 4 types of orders were given (2 of each in each protocol)

1) A visual order — a green light in front of the subject being switched on by a signal from the tape
2) A green light being switched on immediately after a 10 sec count down 10 — 9 — — 1
3) An instruction to the subjects to start pedalling within the following m[illegible]

4) A control order starting with a count down as in 2) which was however not *followed by switching on the green light (i e no work)*

In orders 1) and 2) the green light was the signal to start Following start orders work periods of 1 min were employed throughout In all subjects the results were strikingly similar no matter which starting order 1) 2), or 3) was given and agree well with Dejours s description a rapid rise of ventilation on starting work followed by a plateau lasting some 30 sec and a secondary slower ventilatory rise The study has shown that the initial rapid rise of ventilation on starting work is not a function of the starting order itself when different types of orders are given in random order

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C 8

Effects of Blocking the Autonomic Nervous System during Exercise

By P O ÅSTRAND B EKBLOM and A N GOLDBARG *Department of Physiology Gymnastik & Idrottshogskolan Stockholm Sweden*

The influence of the autonomic nervous system during exercise was investigated in 6 subjects by means of bicycle work (submaximal and maximal loads) during 1) control (C 2) after parasympathetic blockade (PSB) with atropin (2 5 mg iv) 3) after beta adrenergic blockade (BAB) with propranolol (Inderal® 10 mg iv) and 4) after double blockade (DB) with both drugs Maximal V_{O_2} decreased 6 % ($p <$ 0 001) after DB and the time during which the maximal work could be performed was decreased In contrast single blockades did not affect maximal V_{O_2} but during maximal work the time to exhaustion was also reduced after BAB In general resting and submaximal V_{O_2} were not affected by the treatments At rest PSB increased heart rate 60 % (from 65 to 105 beats/min $p <$ 001) but this effect became less pronounced as load increased and maximal heart rate was unchanged BAB decreased resting heart rate 9 beats/min and maximal heart rate 36 beats/min After DB resting heart rate increased it was similar to C at 25 % of maximal V_{O_2} and thereafter progressively decreased compared with C up to maximal work V_E and blood lactate levels during maximal work were reduced after BAB and DB possibly reflecting the decrease of the time during maximal work Both branches of the autonomic nervous system appear to exert similar roles in the setting of the heart rate during exercise at a load of about 40 % of maximal V_{O_2} In order to clarify the circulatory adjustments during work after single and double blockades respectively cardiac output ($\dot{Q}$) by dilution technique and intraarterial blood pressures were measured in *three subjects* After DB and BAB at maximal work $\dot{Q}$ was slightly lower but stroke

volumes were higher PSB did not affect Q during work but stroke volumes were decreased except at maximal load Thus, when heart rate is decreased by either BAB or DB, a compensatory increase in stroke volume takes place to maintain an adequate blood flow to the working muscles

C 9

Effect of Long-term Administration of Thyrocalcitonin on the Skeleton of Calcium-depleted Rats

By O J Malm and L Myhre *Department of Zoophysiology, University of Oslo, Norway*

According to a hypothesis of Hirsch and Munson (1969) thyrocalcitonin (TCT) may serve to protect the skeleton against excessive resorption by parathyroid hormone, especially during periods of calcium deprivation or increased calcium demand

We wished to examine the hypothesis by studying the effect of thyrocalcitonin in dietary osteoporosis *Female and male Wistar rats 6 months old (250 g—500 g)* were divided into 4 groups, one group of treated male rats, one group of control male rats and similar female groups

TCT was administered in a daily dose of 160 MRC milliunits (Ciba porcine Calcitonin) divided into 3 daily subcutaneous injections The control groups received an equal number of injections of sterile isotonic saline solution The experiment lasted 3 months

TCT treated rats showed an increase in the dry weight of femur, but specific gravity and calcium, phosphorus and magnesium contents failed to show a marked change For the caudal vertebrae similar results were found for mineral contents

Table I Dry weight and mineral contents of femur, mean ± standard deviation Each group consists of nine rats Calcium, magnesium and inorganic phosphorus determinations were made on pooled samples of serum

		Rats treated with TCT	Control	Significance
Dry weight of femur, gram	♂	0 647 ± 0 015	0 626 ± 0 029	p < 0 01
	♀	0 358 ± 0 017	0 315 ± 0 018	p < 0 01
% Ca in femur	♂	24 2 ± 0 5	24 5 ± 0 4	p > 0 5
	♀	24 1 ± 0 5	24 5 ± 0 8	p > 0 5
% P in femur	♂	13 9 ± 0 5	13 0 ± 0 9	p > 0 5
	♀	12 2 ± 0.2	15 1 ± 0 9	p > 0 5
% Mg in femur	♂	0 48 ± 0 01	0 49 ± 0 01	p > 0 5
	♀	0 48 ± 0 02	0 54 ± 0 02	p < 0 005
Ca meq/l serum	♂	4 90	5 00	
	♀	4 80	5 20	
Mg meq/l serum	♂	2 24	2 69	
	♀	2 20	2 37	
P mg/100 ml serum	♂	4 36	3 92	
	♀	3 43	3 04	

Serum analysis showed slight decrease in calcium and magnesium concentration and slight increase in inorganic phosphorus concentration in the treated rats The results are summarized in Table I

The slight hypocalcemic effect of thyrocalcitonin may be due to a compensatory hypersecretion of parathyroid hormone in the calcium depleted rats Foster *et al* (1966) found a similar difference between normal and parathyroidectomized rats suggesting TCT stimulated hypersecretion of parathyroid hormone in normal rats

The experiment indicates that in our calcium deprived rats TCT may preserve bone mass without changing the bone mineral concentrations Klein and Talmage (1968) showed that TCT decreased the rate of removal of hydroxyproline from bone both in intact and parathyroidectomized rats This finding together with our results may indicate that TCT also influences the collagen component of bone

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C 10

Central Regulation of ADH release in the Conscious Goat

By L ERIKSSON* O FERNANDEZ** and K OLSSON *Department of Physiology Veterinarhogskolan 104 05 Stockholm 50 Sweden*

The antidiuretic response to intracarotid infusions of hypertonic sodium salts and sucrose in the hydrated dog suggests that hypothalamic osmoreceptors regulate the release of antidiuretic hormone (ADH) (Verney 1947) However hypertonic solutions of di or monosaccharides infused into the 3rd ventricle of the goat have no antidiuretic effect although similar infusions of hypertonic NaCl effectively release ADH (Andersson *et al* 1967 Olsson 1969) Therefore it appeared of interest to study the effect of these osmotic stimuli when applied outside the blood brain barrier in the hydrated goat

Vinyl catheters were implanted into the carotid artery via its facial or superficial temporal branches Various solutions were infused through these catheters into the blood supply of the brain in the conscious undisturbed animal during hydration Vinyl catheters implanted into the jugular vein via its superficial temporal branch were used for control experiments

Prolonged intra arterial (10—30 min 1 5 ml/min or 2 ml/min) infusions of hypertonic solutions of NaCl (0 5 M or 1 M) and of sucrose fructose glucose and galactose were made The infusions of hypertonic NaCl or sucrose invariably turned renal free water clearance into negativity for at least half an hour indicating a release of

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ADH Of the monosaccharides, only fructose gave a similar antidiuretic response I v infusion (30 min 1 5 ml/min) of NaCl (0 5 M) and of fructose (1 M) had no or very little antidiuretic effect

It is likely that in each animal infusions of equi-osmolar solutions caused approximately the same increase in the osmotic pressure of the blood reaching the brain However, investigations performed in other species have revealed different penetration rates across the blood brain barrier for the substances used here Thus a transport mechanism facilitates the brain uptake of glucose, whereas a rather effective blood brain barrier seems to exist for sucrose fructose and sodium ions (*cf* Crone 1965 Brooks *et al* 1970) The latter three substances had appreciable antidiuretic effect in the goat This suggests that brain barrier systems are of greater importance in the osmotic regulation of the ADH release than the membrane permeability of certain cells in the hypothalamus

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C 11

A Mucopolysaccharide Protein Complex with Amine Binding Properties in Rat Thrombocytes

By C H Åborg and B Uvnäs *Department of Pharmacology Karolinska Institutet Stockholm Sweden*

Trombocyte granules store histamine (Hi) and 5 hydroxytryptamine (5 HT) The granules contain large quantities of adenosine triphosphate (ATP) The nucleotide is generally assumed to be essential for the storage of the amines the assumption being founded on the fact that *in vitro* ATP forms a complex with 5 HT (Da Prada and Pletscher 1968) as well as with Hi (Schauer and Eder 1961)

The amine binding properties of the heparin protein complex of mast cell granules led us to look for a similar complex in rat thrombocyte granules Such granules were obtained by sonication of thrombocyte rich plasma fractions suspended in isotonic sucrose After removal of coarse debris by centrifugation at 2000×*g* and recentrifugation of the supernatant at 18 000×*g* granule containing fractions were resuspended in small volumes of deionized water undissolved material spun down at 3000×*g* resuspended and recentrifuged and then used either as such or after drying *in vacuo* subsequent washing in chloroform to remove lipids and final drying All procedures were performed as close to 0° C as possible When the insoluble granule material was resuspended in water solutions containing 5 HT Hi or sodium with admixture of ^{3}H 5 HT, ^{3}H Hi or ^{22}Na re

Rats were given $Na_2{}^{35}SO_4$ 5 mCi subc on each of two consecutive days and blood was drawn on the third day The thrombocytes were charged with ^{3}H 5 HT or ^{3}H Hi by incubating plasma from 6 rats with ^{3}H 5 hydroxytryptophane or ^{3}H histidine respectively (0 25 mCi) for 45 min at 37° C and thrombocyte granules prepared as described above When centrifuged through the Ficoll gradient the distribution of the ^{35}S and the labelled amines coincided On dissolution of the amine binding granule material in 1 M NaCl solution and subsequent passage through a Dowex I X2 the material separated into two fractions one protein fraction in the void volume and one ^{35}S containing fraction eluted with 5 M NaCl As demonstrated on disc gel electrophoresis the former fraction contained basic proteins The latter fraction contained hexose amine which was tentatively shown to originate from chondroitine sulphuric acid

The results have demonstrated the presence in rat thrombocyte granules of a mucopolysaccharide protein complex capable of binding Hi 5 HT and Na Calculations showed that the binding capacity of this complex sufficed for the storage of the quantities of the amines (5 HT and Hi) which normally occur in rat plasma thus as previously found to be the case in mast cell granules the possibility exists that also in the thrombocyte granules the biogenic amines are stored in ionic linkage with a mucopolysaccharide protein complex and not with ATP as currently assumed

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C 12

Effect of Acetylcholine on the Efflux of Calcium 45 from the Perfused Cat Submandibular Gland

By S P NIELSEN and O H PETERSEN *Institute of Medical Physiology C, University of Copenhagen Denmark*

The importance of extracellular calcium for salivary secretion is well established (Douglas and Poisner 1963) Stimulation of salivary secretion is initially accompanied by a large transport of calcium into the saliva (Nielsen and Petersen 1970) It is unknown whether acetylcholine has an effect on calcium transport across the contraluminal cell membrane The cat submandibular gland was labelled with 20 μCi Ca^{45} given as a 1 hr close-arterial infusion retrogradely into the lingual artery Thereafter the gland was perfused at a constant flow with a non radioactive oxygenated Locke solution and the efflux of Ca^{45} from the cells to the perfusion fluid was studied

A close arterial injection of 0 06 μmole acetylcholine was followed by a pronounced rise in the efflux of Ca^{45} An enhanced concentration of Ca^{45} in the effluent was present in the sample collected during the first period (20 sec) after the stimu

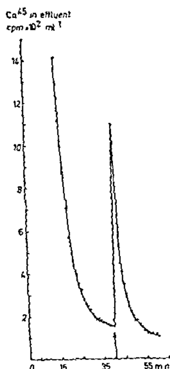


Fig 1 Effect of 0 06 μmole acetylcholine (↑) on the efflux of Ca^{45} from the pre-labelled submandibular gland

lation Fig 1 shows a typical experiment The effect of 0 06 μmole adrenaline was indistinguishable from that of acetylcholine Perfusion for 15 min with a Locke solution equilibrated with nitrogen with or without the addition of 10^{-3} M mono-iodoacetate did not seem to affect the acetylcholine induced Ca^{45} efflux

It has previously been shown that acetylcholine increases the permeability of the contraluminal acinar cell membrane to sodium and potassium (Petersen 1970) The present results suggest that acetylcholine augments the calcium permeability of the contraluminal cell membrane

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C 13

Function of Single Interneurones Established by their Monosynaptic Inhibitory Effects on Motoneurones

By E Jankowska and W Roberts *Department of Physiology, University of Göteborg, Sweden*

Hultborn *et al* (1971) postulated that the inhibition of motoneurones by activity in group Ia afferents from antagonist muscles is mediated by Ia interneurones located in the ventral horn Their axonal projection to antagonist motor nuclei has been demonstrated, it was shown that ventral horn interneurones excited from quadriceps *(Q) group Ia afferents can be antidromically activated from distinct loci within* posterior biceps *(PB)* and semitendinosus *(St)* motor nuclei (Jankowska and Roberts 1971) The present experiments provide evidence that impulses in these interneurones produce monosynaptic inhibitory postsynaptic potentials (IPSPs) in PB and St motoneurones

The experimental procedure is illustrated in Fig 1 First, extracellular records were taken from a proper interneurone in L5—L6 segments with a glutamate filled microelectrode (diagram I) Current pulses were passed through a second microelectrode (filled with citrate, shielded, *cf* Eide 1971) which was moved about in the

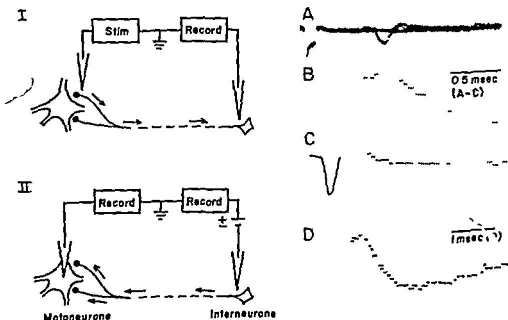


Fig 1 I and II are diagrams of the experimental arrangement (see text) A Extracellular

PBSt motor nucleus in L7 or S1 until a point was found from which the interneurone could be antidromically activated (record A) with a threshold below 3 μA The citrate electrode was then used to record intracellularly from nearby motoneurones (diagram II) The interneurone was made to fire repetitively by the ejection of glutamate ions and its spikes were used to trigger a computer which averaged the intracellular potentials recorded from the motoneurones An example of the IPSPs following interneuronal spikes is given by records B and D The latencies of the IPSPs with respect to the interneuronal spikes (C) were 0 30—0 40 msec longer than the conduction times from the interneurone axon terminals to their somas, indicating a synaptic delay of about 0 3 msec The IPSPs ranged in amplitude from 10 to 250 μV and were found in a majority of the motoneurones impaled within 150 μ of the low threshold loci The latter finding demonstrates that each interneurone makes synaptic contact with a great number of motoneurones

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C 14

Two Pathways from the Mesencephalon to Fusimotor Neurones of the Contralateral Flexor Digitorum Longus Muscle of the Cat

By B APPELBERG and T JENESKOG *Department of Physiology, Faculty of Medicine, University of Umeå, Sweden*

In cats anesthetized with halothane two mesencephalic regions were found to influence extensor fusimotor neurones From a dorsal region augmentation of spindle dynamic sensitivity was elicited At the level of the caudal red nucleus this region corresponded well to the rubral antidromic field potential Caudally to the red nucleus however, no antidromic response could be seen, but the stimulating effect was still obtained at a low threshold The descending pathway from the dorsal region was interrupted by lesions in the dorsal part of the contralateral lateral funiculus

From the ventral region static or a mixture of static and dynamic effects were elicited This region corresponded well to the fibre bundle crossing the midline just dorsal to the interpeduncular nucleus The pathway from the ventral region descended in ventral and lateral parts of the contralateral funiculus

Sometimes the two regions were separated by a region with higher thresholds to stimulation on other occasions they were confluent A mixture of effects from the two regions where then often obtained Under such circumstances however, critical spinal lesions could separate between the effects caused by the two systems This is shown in Fig 1 where a mixed static/dynamic influence upon the spindle in B is changed to a purely dynamic effect in D by a ventro-lateral spinal lesion

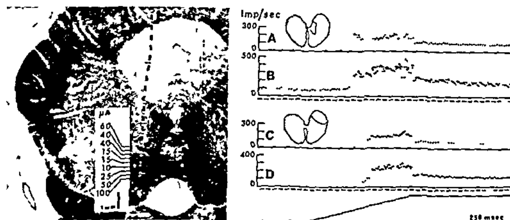


Fig 1 *Left* Transverse section through the mesencephalon 1 mm caudally to the red nucleus Inset showing stimulating thresholds for mixed static/dynamic effect on spindle at different depths along electrode track

Right Instantaneous frequency plots of spindle response to 4 mm extension of muscle at 8 mm/sec (oblique line below records) A, control contralateral spinal half intact B stimulation 300/sec, 50 µA in point of lowest threshold C, control, ventral and lateral parts of spinal half cut D, stimulation 60 µA

Effects from the two regions could be obtained after cerebellectomy and also in cats with degenerated cortico-spinal tracts

With regard to the lack of agreement between the dorsal region and the red nucleus caudal levels it is suggested that the efferent pathway is not identical to the rubro-spinal tract The influence of the inferior olivary region on dynamic fusimotor neurones should be recalled (Appelberg and Molander 1967) The ventral region is likely to represent the outflow from the mesencephalic reticular formation (Vedel and Mouillac Baudevin 1969)

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C 15

Smooth Muscular Hypertrophy of a Medium-Sized Artery Following a Local Drop in Pressure and an Enormous Increase in Blood Flow

By R D Harkness, R Ingebrigtsen and L A Solberg *Institute for Experimental Medical Research, University of Oslo, the Department of Pathology, Ullevål Hospital, Oslo, Norway and the Department of Physiology, University College, London, England*

In 19 dogs, hypertrophy of the femoral artery proximal to an arterio-venous fistula was studied in histological van Gieson-stained sections and compared with the femoral artery of the control side After autopsy and excision, the pieces were submitted to

fixation in formalin From another series of 5 dogs the pieces were submerged in and filled with 10 % formalin under a pressure of 190 cm water for 24 h The transverse area of the median coat of the arteries was measured planimetrically in magnified projections of the sections, and the difference in size between the fistulous and control vessels was calculated percentually to the smallest area

The weight of equal lengths of the two arteries was determined after excision and removal of adventitia Dry weight of some pieces was also measured Such pieces were submitted to biochemical quantitative analysis of elastin and collagen.

The drop in pressure and the enormous increase in flow produced an immediate increase of 10 % in the diameter of the artery proximal to the fistula In 12 of the 19 dogs of the first series, the transverse area of the median coat increased from the 3rd month after creation of the fistula up to the 15th month when it had increased by 100 % From now on up to the 52nd month the increase was rather small in 3 dogs and in 4 dogs it was negative *i e*, an atrophy had occurred, as is well known from human patients From the 2nd month, the weight of the pieces of the fistulous artery increased with some spreading up to the stage of weighing at 19 months after creation of the fistula, when it had increased by 105 % Dry weight of the two arteries was also examined up to the 11th month when the weight had increased by 35 % The 5 pairs of arteries submitted to fixation under pressure showed that the proportion of muscle tissue in the median coat of the fistulous artery had increased compared to the other side Chemical analysis of elastin and collagen showed very little difference between the normal and the fistulous artery

Conclusion

In a medium sized artery a local fall of pressure and a large increase in blood flow will induce hypertrophy of the muscular elements of the median coat

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C 16

The Effects of Immunosympathectomy on Blood Pressure and Vascular "Reactivity in Normal and Spontaneously Hypertensive Rats

By B FOLKOW M HALLBACK Y LUNDGREN and L WEISS *Department of Physiology University of Goteborg Sweden*

Newborn litters of spontaneously hypertensive rats (SHR) and normotensive control rats (NCR) were given identical injections of sympathetic nerve growth factor antiserum (Burroughs Wellcome) resting blood pressure being intermittently recorded

for 8 months These "immunosympathectomized" rats (SHR_{IS} and NCR_{IS}) had clearly lower blood pressures than ordinary SHR and NCR, SHR_{IS} being in this respect almost equal to NCR but showing a significant, nearly 25 % higher pressure than NCR_{IS}

In 20 paired hindquarter perfusions, one SHR_{IS} or NCR_{IS} was compared to one untreated NCR during constant flow conditions, from the level of complete vascular relaxation up to maximal resistance responses as induced by graded noradrenaline (NA) doses Besides a moderate shift to the left of the "resistance curves" of NCR_{IS} and particularly of SHR_{IS}, reflecting their denervation hypersensitivity, the treatment had altered the characteristics of the SHR_{IS} curves towards those of ordinary NCR. Thus, among the highly significant increases of resistance at maximal dilatation, of curve steepness and of maximal pressor response in ordinary SHR as compared with NCR (Folkow *et al* 1970 a, b) only the two latter ones were still evident in SHR_{IS} and to a reduced extent These results indicate that a marked reduction of the adrenergic excitatory impact, as caused by immunosympathectomy, correspondingly delimits the considerable structural vascular adaptation ordinarily present in SHR

However, on relating the modest but significant blood pressure difference between SHR_{IS} and NCR_{IS} (nearly 25 %, compared with about 50 % in ordinary SHR and NCR) to the corresponding differences in resistance at maximal dilatation, in curve steepness and in maximal pressor response, all these parameters differed significantly between SHR_{IS} and NCR_{IS} and largely in proportion to their pressure difference This indicates that the structurally based adaptation of the resistance vessels that distinguishes ordinary SHR from NCR (Folkow *et al* 1970 a, b) can still be traced when SHR_{IS} are compared with NCR_{IS}

If for genetical reasons the average sympathetic activity is enhanced in SHR, these indications of a structural vascular adaptation also in SHR_{IS} might, of course, be a simple consequence of such a functional drive being then conveyed via residual neuro-hormonal connections since a complete sympathetic destruction is unlikely to have occurred in SHR_{IS}—NCR_{IS} On the other hand, the tendency of a stronger NA sensitization in SHR_{IS} than in NCR_{IS} may reflect a more efficient sympathectomy in SHR_{IS} and hence a relatively less efficient neurohormonal control in these animals Therefore the possibility remains that the blood vessels, and mesenchymal tissues in general, are somewhat more prone to display structural adaptations to given loads in *SHR, implying that their massive hypertension may be genetically* linked to both a functional and a structural element

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C 17

Effect of Prostaglandin $F_{2\alpha}$ on Airway Resistance in Man

By P HEDQVIST, A HOLMGREN and A A MATHE. *Departments of Physiology, Karolinska Institutet and Clinical Physiology, Karolinska Sjukhuset, Stockholm, Sweden*

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is a normal constituent of lung tissue in many animal species including *homo* (Anggård 1965) $PGF_{2\alpha}$ is released in the anaphylactic reaction of the guinea pig lung (Piper and Vane 1969) but its possible role in the allergic disorders is as yet unclear Isolated bronchi from guinea pig cat and man contract in the presence of $PGF_{2\alpha}$, and in guinea pig and cat i v administration of $PGF_{2\alpha}$ increases airway resistance indicating that the compound may have a similar action *in vivo* (Änggård and Bergstrom 1963, Berry and Collier 1964, Mathe Strandberg and Åstrom 1971)

On the basis of these observations it was decided to study the effect of $PGF_{2\alpha}$ on human lungs *in vivo* Airway resistance (AWR) was measured in 10 healthy volunteers by means of whole body plethysmography and was conventionally expressed as airway conductance (SG_{aw}) Isotonic sodium chloride histamine and $PGF_{2\alpha}$ were administered by inhalation of an aerosol mist produced by an ultrasonic nebulizer AWR was measured before and 1 5 and 10 min after each aerosol inhalation, the value obtained at each step representing the mean of 5 consecutive readings

Inhalation of isotonic sodium chloride ($PGF_{2\alpha}$ and Hi solvent) did not materially affect the SG_{aw} whereas $PGF_{2\alpha}$ produced a decrease in SG_{aw} One minute after inhalation of $PGF_{2\alpha}$ the mean decrease of SG_{aw} was with 64 μg 14 %, with 256 μg 21 % with 512 μg 31 % and with 1024 μg 49 % The effect of $PGF_{2\alpha}$ was relatively shortlasting and complete recovery occurred within 10 min. Approximately 3 times higher doses (on a molar basis) of histamine were required to induce a comparable decrease in SG_{aw}

From the results reported in this communication it may be concluded that $PGF_{2\alpha}$ is a potent bronchoconstricting agent in the human lung *in vivo* The doses needed to produce this effect are fairly low considering that only a small fraction of the aerosol will reach the reactive sites in the lung The natural occurrence availability for release and demonstrated effects upon application merit further investigation of the role of $PGF_{2\alpha}$ in normal lung function and in certain lung disorders such as bronchial asthma

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C 18

A Latency in the Activation of Lipolysis in Adipose Tissue during Nerve Stimulation

By B B FREDHOLM *Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden*

Following stimulation of the nervous supply to canine subcutaneous adipose tissue an increased rate of free fatty acid (FFA) and glycerol release is observed (Rosell 1966 Fredholm and Rosell 1968) The increased release rate is not seen immediately upon the start of the stimulation and the highest release rates generally occur after the cessation of the stimulation This pattern of response is changed by adrenergic α receptor antagonists *e g* dihydroergotamine the rate of glycerol and FFA release increases rapidly upon nerve stimulation and the highest release rates are generally seen during the stimulation period Moreover, the amount of FFA and glycerol released is higher after α blockade (Fredholm and Rosell 1968)

There were essentially three different possibilities to explain these findings 1 Stimulation of adrenergic α receptors counteracts the lipomobilizing effect of adrenergic β receptor stimulation 2 The lipolytic process starts immediately upon the initiation of nerve stimulation but the outflow of the products of lipolysis is delayed owing to trapping in the tissue secondary to vasoconstriction 3 Not only the outflow of lipolytic products but the activation of lipolysis is also delayed

The first possibility was tested in six experiments where the rate of glycerol and FFA release was increased by theophylline (Fredholm 1970) and the β receptors inhibited by propranolol (400 mg i a) During nerve stimulation after this pretreatment there was a decreased glycerol outflow but after cessation of the stimulus a transient increase was observed There was no net effect of the nerve stimulation indicating that there is no significant inhibition of lipolysis by adrenergic α stimulation

In an attempt to discriminate between the two remaining possibilities a series of eight experiments were carried out Two consequtive nerve stimulations (4 cps for 20 min) were applied During the second propranolol was given after 12 min If the animals were pretreated with dihydroergotamine the second nerve stimulation caused the release of 89 ± 23 per cent of the first If on the other hand no pretreatment was given the figure was 34 ± 12 per cent It can be concluded that if the adrenergic α receptors are intact significantly smaller lipolysis occurred during the first part of the stimulation compared to the latter

The results thus demonstrate a latency in the activation of lipolysis during nerve stimulation in canine subcutaneous tissue It is conceivable that a severed transport of the adrenergic transmitter substance from nerve ending to fat cell is the underlying cause (Fredholm and Rosell 1970)

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C 19

Hormonal Regulation of Ovarian Phosphorylase Activity

By K Ahren and G Selstam *Department of Physiology, University of Göteborg, Sweden*

Luteinizing hormone (LH) is known to increase glucose uptake and lactate production by isolated ovaries from prepubertal rats Follicle stimulating hormone (FSH) has been reported to have similar effects, but this gonadotropin showed another dose response curve with the lowest effective dose approximately 100 times higher than for LH (Hamberger and Ahren 1967) The effect of LH on glucose uptake has been shown to be a direct stimulation of membrane transport (Ahren and Hamberger 1969) In order to see whether there are also endogenous sources for the increased lactate production after gonadotropic stimulation, the effect of gonadotropins on phosphorylase activity was studied

Dissection and incubation procedures of prepubertal rat ovaries were performed as described earlier (*e g* Hamberger and Ahren 1967) Phosphorylase was determined according to Bueding *et al* 1962 As in other tissues, the phosphorylase enzyme was found to consist of two forms, phosphorylase *a* and phosphorylase *b*, where the former is active in the absence of 5′ AMP and the latter requires 5′-AMP for activity Table I shows that both LH and FSH increased phosphorylase activity, *i e* increased per cent phosphorylase *a* Dibutyryl (cyclic) adenosine 3′,5′ monophosphate (3′,5′-AMP) was tested in concentrations known to stimulate lactate production (Herlitz *et al* 1971), and this nucleotide markedly stimulated phosphorylase activity Addition of adrenaline had no effect in concentrations up to 1 μg/ml while a concentration of 10 μg/ml increased per cent phosphorylase *a* (Table I) The absolute activity of phosphorylase *a* + *b* was not changed in any of the experiments

Table I Effects of LH FSH, 3 , 5 AMP and adrenaline on phosphorylase activity in the prepubertal rat ovary[1]

		Per cent phosphorylase *a*		
		Control	Hormone	Signif of effect
LH	100 μg/ml	22 0±1 7	29 6±0 8	p < 0 01
	10 μg/ml		28 9±0 8	p < 0 01
	1 μg/ml		23 3±1 7	p > 0 4
FSH	100 μg/ml	17 6±1 8	28 1±0 6	p < 0 001
	10 μg/ml		23 3±2 2	p < 0 05
	1 μg/ml		19 3±1 1	p > 0 3
3 , 5 AMP	25 mM	18 4±0 8	39 5±2 2	p < 0 001
	10 mM		40 8±3 3	p < 0 001
	1 mM		26 5±0 6	p < 0 001
Epinephrine	10 μg/ml	25 1±0 9	34 7±2 2	p < 0 005
	1 μg/ml		27 2±1 8	p > 0 3

[1] Ovaries were incubated for 30 m[illegible]
37° C and pH [illegible]
ing to the me[illegible]
in absence (ph[illegible]
glucose 1 phos[illegible]

It is thus clear that gonadotropins can increase ovarian phosphorylase activity LH and FSH seem to have quantitatively very similar effects The fact that 3,5′ AMP had a marked effect on phosphorylase activity of the prepubertal rat ovary is of particular interest in relation to the report that this nucleotide did not influence phosphorylase activity of bovine corpus luteum where LH had an effect (Marsh and Savard 1964)

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C 20

Interactions between Sugar and Amino Acid Transport in Rat Jejunum

By B G Munck *Inst Med Physiol A, University of Copenhagen Denmark*

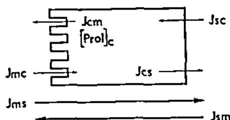
Actively transported nonmetabolized sugars inhibit intestinal amino acid transport (Newey and Smyth 1964) This has been interpreted in terms of a multifunctional carrier common to sugars and amino acids (Alvarado 1966 1970 Alvarado *et al* 1970) although other experiments suggest that this inhibition results from increased efflux across the brush border (Chez *et al* 1966 Munck 1968)

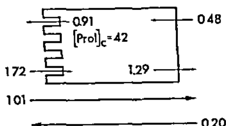
The unitarian hypothesis is based on measurements of amino acid uptake by pieces of intestine including all layers or by sacs of everted intestine Neither method allows measurements or estimates of transmembrane fluxes or intraepithelial concentrations Sugar amino acid interactions have therefore been reinvestigated Methods (Munck and Schultz 1969) were used which allow precise characterization of fluxes across the brush border membrane estimates of fluxes across the contraluminal membrane, and measurements of steady state epithelial accumulation Test solutions were Krebs phosphate buffer with 10 mM l proline + 28 mM mannitol or 10 mM l proline + 28 mM d galactose (pH 7 4 37° O aeration)

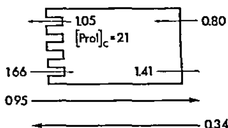
The figure (idealized epithelial cells) defines the symbols It contains measured and calculated data fluxes in μmoles/hr cm² concentrations in mM The measured values are given below for Prol + Man /Prol + Gal as means with s e and number of observations

J_{mc} 1 72±0 20 (6)/1 66±0 15 (6) J_{ms} 1 01±0 33 (4)/0 95±0 19 (5) J_{sm} 0 20±0 04 (5)/0 34±0 04 (5), [Prol]$_c$ 42±2 (24)/21±1 (24)

These data show that galactose inhibits epithelial uptake and transepithelial net transport of proline by increasing J_{cm} and J_{sm} without interference with J_{m} That is they are inconsistent with the notion of a common carrier for sugar and amino acids







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C 21

Gastric Acid Response to Sham Feeding in Duodenal Ulcer Patients before and after Antrectomy

By U KNUTSON and L OLBE *Department of Surgery II, Sahlgrenska sjukhuset, Göteborg, Sweden*

Hypersecretion of gastric acid juice is considered of great importance in the genesis of duodenal ulcers in man Dragstedt has postulated that this hypersecretion is caused by vagal hyperactivity Experiments in animals have shown that the acid response to physiological vagal activation (sham feeding) is the effect of a direct vagal action on the acid secreting glands that is potentiated by a simultaneous vagal release of the antral hormone gastrin

A sham feeding procedure in man has been evolved Sham feeding implies a normal feeding with the exception that the food never reaches the stomach The method is applicable in pept[illegible]

It is thus clear that gonadotropins can increase ovarian phosphorylase activity LH and FSH seem to have quantitatively very similar effects The fact that 3′,5′-AMP had a marked effect on phosphorylase activity of the prepubertal rat ovary is of particular interest in relation to the report that this nucleotide did not influence phosphorylase activity of bovine corpus luteum where LH had an effect (Marsh and Savard 1964)

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C 20

Interactions between Sugar and Amino Acid Transport in Rat Jejunum

By B G MUNCK, *Inst Med Physiol A, University of Copenhagen, Denmark*

Actively transported nonmetabolized sugars inhibit intestinal amino acid transport (Newey and Smyth 1964) This has been interpreted in terms of a multifunctional carrier common to sugars and amino acids (Alvarado 1966 1970, Alvarado *et al* 1970) although other experiments suggest that this inhibition results from increased efflux across the brush border (Chez *et al* 1966, Munck 1968)

The unitarian hypothesis is based on measurements of amino acid uptake by pieces of intestine including all layers or by sacs of everted intestine Neither method allows measurements or estimates of transmembrane fluxes or intraepithelial concentrations Sugar amino acid interactions have therefore been reinvestigated Methods (Munck and Schultz 1969) were used which allow precise characterization of fluxes across the brush border membrane estimates of fluxes across the contraluminal membrane, and measurements of steady state epithelial accumulation Test solutions were Krebs phosphate buffer with 10 mM l proline + 28 mM mannitol or 10 mM l proline + 28 mM d galactose (pH 7 4 37 O_2 aeration)

The figure (idealized epithelial cells) defines the symbols It contains measured and calculated data fluxes in μmoles/hr cm² concentrations in mM The measured values are given below for Prol + Man /Prol + Gal as means with s e and number of observations

J_{mc} *1 72 ± 0 20 (6)/1 66 ± 0 15 (6* J_{ms} *1 01 ± 0 33 (4)/0 95 ± 0 19 (5),* J_{sm} *0 20 ± 0 04 (5)/0 34 ± 0 04 (5),* $[Prol]_c$ *42 ± 2 (24)/21 ± 1 (24)*

These data show that galactose inhibits epithelial uptake and transepithelial net transport of proline by increasing J_{cm} and J_{sm} without interference with J_{mc} That is they are inconsistent with the notion of a common carrier for sugar and amino acids

mechanical constriction of the inferior caval vein, the ratios were similar Experiments in anesthetized, open chest dogs and in conscious dogs with implanted ultrasonic elements and an electromagnetic flowmeter on the ascending aorta showed no consistent differences Similar responses were obtained from the apical region and at the base of the left ventricle Loading was also performed by infusing isothermal whole blood at the same rates to exclude interference to cardiac responses caused by *hemodilution or possible inotropic effects of saline* The interrelations between MCL, SV and left ventricular end-diastolic pressure were not significantly different whether saline or blood was infused It is concluded that 1) MCL recording can be used for translating myocardial mechanical events into changes in stroke volume, 2) ESMCL is better maintained during variations in SV in experiments performed at high adrenergic activity, this mechanism improves emptying of the distended ventricle

C 23

Effects of Selective Arm- and Leg Training on Cardiac Output and Regional Blood Flow

By J P CLAUSEN, K KLAUSEN, B RASMUSSEN and J TRAP-JENSEN *Department of Clinical Physiology, Bispebjerg Hospital and Laboratory for the Theory of Gymnastics, University of Copenhagen, Denmark*

In a previous study (Clausen, Trap-Jensen and Lassen 1970) it was found that training of the arm muscles caused a very pronounced reduction in heart rate during submaximal arm work This reduction could not be transferred to work performed with the nontrained leg muscles The aim of the present investigation was to study whether other circulatory changes induced by training are confined to work performed with the trained muscle groups

10 young healthy subjects trained daily on bicycle ergometer during 5 weeks Five trained the arm muscles, and five trained the leg muscles Before and after the training hemodynamic data were obtained in all subjects during arm as well as during leg exercise at a moderate and heavy work load

Arm training During arm work at the moderate and heavy load the effect of training was a very pronounced reduction in heart rate of 13 3 % and 16 8 % ($\Delta = -18$ and -28 beats/min) and a reduction in cardiac output of 10 7 % and 8 9 % ($\Delta = -1$ 42 and -1 50 l/min) The oxygen uptake decreased by 8 4 % and 6 4 % ($\Delta = -105$ and -112 ml/min) The arterio-venous oxygen difference (a v O diff) from the working arms increased at both loads 6 8 % and 12 2 % (Δ = 7 9 and 14 1 ml O_2/l) Hepatic blood flow (HBF) was increased (Δ = 6 6 % and 16 4 %) The only effect of arm training which was transferred to some extent to work performed with the non trained leg muscles was a decrease in heart rate of 6 % and 4 % ($\Delta = -8$ and -6 8 beats/min) whereas cardiac output, oxygen uptake a v O_2 diff in the working leg and HBF were unchanged

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Leg training During leg exercise the effects of training were a reduction in heart rate 9 3 % and 8 7 % ($\Delta = -13$ and -15 beats/min) at the two work loads Cardiac output was 12 % (1 71 l/min) lower at the moderate load, but unchanged at the heavy load The a v O_2 diff for the working legs was not changed HBF increased at both loads ($\Delta = 7$ 7 % and 10 0 %) During work with the non trained arms heart rate was reduced 10 5 % and 7 8 % ($\Delta = -13$ beats/min at both loads) *i e* a reduction similar to that found for work performed with the trained leg muscles Cardiac output was unchanged at the moderate arm load but increased 7 4 % (1 2 l/min) during heavy arm work No significant changes occurred in a v O_2 diff or in HBF during arm exercise

These results suggest that the very pronounced reduction in heart rate obtained by arm training depends primarily on local changes in the trained arm muscles Leg training seems in addition to modify the central circulatory adaptation to exercise also when the work is performed with untrained muscles

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C 24

Effect of Acetylcholine on the Electrical and Mechanical Responses of the Guinea-pig Vas Deferens to Nerve Stimulation

By N O Sjostrand* *University Department of Pharmacology Oxford England*

Low concentrations of acetylcholine (Ach) potentiate the motor response of the guinea pig vas deferens to nerve stimulation (Sjostrand 1961 Sjostrand and Swedin 1968) In order to obtain more detailed information on this phenomenon a study on the electrical as well as the mechanical responses of the organ has been performed The sucrose gap technique was used The preparation and conditions were essentially the same as those of Burnstock *et al* 1964

Ach (10^{-9}—10^{-6} g/ml) produced a depolarization of the smooth muscle During this depolarization the excitatory junction potentials (EJP) evoked by nerve stimulation diminished However in general the depolarization caused by the EJP s and Ach together was larger than that caused by the EJP s alone Because of this EJP s which were too small to reach the threshold for firing an action potential (AP) in the presence of Ach could elicit an AP Or in the case of summating EJP s fewer EJP s were required to obtain an AP However when the Ach induced depolarization was large and the EJP s initially were minute sometimes more EJP s were needed Prolonged exposure to Ach (2—15 min) gradually increased the threshold in some preparations

* Present address Department of Physiology Karolinska Institutet Stockholm Sweden

The appearance of the compound propagated AP also changed in the presence of Ach Generally it became larger and had a shorter duration Simultaneously the contractions became larger This change in spike configuration and size of contraction persisted also after a prolonged exposure to Ach Atropine (10^{-6} g/ml) completely abolished all effects of Ach

The results indicate that the main action of Ach is a depolarization of the smooth muscle cell membrane This presumably affects the vas deferens in two ways it increases the excitability of the organ to nerve stimulation It also influences the propagation of action potentials in the tissue, enlarges the involved cell area and probably also increases the speed of the excitation wave The sum of these events will be that more cells are engaged in the contraction Finally it cannot be excluded that Ach increases the flux of calcium ions into the cells and thereby affects the contraction processes The result does not, however, indicate that Ach to any marked extent affects the nervous transmission in the organ.

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C 25

Repetitive Impulse Firing Properties of a Neurone Model Based on "Voltage Clamp Equations" Compared to the Properties of Spinal Motoneurones

By D KERNELL and H SJOHOLM *Nobel Institute for Neurophysiology Karolinska Institutet, Stockholm, Sweden*

For the understanding of the integrative functions of central neurones it is important to know how their firing rate may be controlled by maintained currents such as those produced by asynchronous synaptic activity Repetitive impulse discharges elicited by steady currents have been studied in several recent works on spinal motoneurones (*e g* Granit Kernell and Shortess 1963 Kernell 1966 1970) For the interpretation of findings from such experiments it would be desirable to know the properties of the motoneurone membrane (*e g* the dependence of ionic permeabilities on membrane potential and time) Precise knowledge of this kind is available for certain peripheral nerve fibres but not for spinal motoneurones In the present work we have used a neurone model for studies concerning the importance of various membrane properties for repetitive impulse firing The neurone model was based on the Frankenhaeuser—Huxley equations (1964) for voltage clamp data from the amphibian peripheral nerve Repetitive impulse firing was elicited by constant stimulating currents The calculations were made with a digital computer

With the original standard data of Frankenhaeuser and Huxley (1964), the repetitive impulse firing of the neurone model differed markedly from that of cat spinal motoneurones Thus, in this version of the neurone model, firing rates below 100 impulses/sec could not be elicited by constant current, the curve relating firing rate to the intensity of stimulating current (the 'f I curve') showed an upward convexity, and there was generally no initial adaptation of firing rate just after the abrupt onset of a constant stimulating current (the initial impulse interval was briefer than the succeeding ones only at the very weakest current intensities)

The equations were then modified (mainly by changes in various constants) to make the model conform more closely to certain known membrane properties of cat spinal motoneurones Thus, for instance, changes were made that concerned the afterpotentials, the membrane time constant, and the 'accomodative' effects of subthreshold depolarizations Following appropriate modifications of this kind the repetitive behaviour of the neurone model became similar to that of spinal motoneurones from several points of view Further computations indicated that the permeability mechanisms underlying the after hyperpolarization (due mainly to potassium permeability) were of great importance for the repetitive behaviour of the neurone model (*e g* for its ability to fire at slow rates, for the shape and slope of the f I curve, and for the initial adaptation of firing rate after the abrupt onset of constant stimulation)

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C 26

Spectral Sensitivity of the Compound Eye in Three Heliconius Species

By G STRUWE *Department of Physiology, Karolinska Institutet Stockholm Sweden*

Behavioural and electrophysiological experiments indicate that insects generally have poor sensitivity to light at wavelengths longer than 600 nm From behavioural experiments, however it is known that the tropical butterfly *Heliconius erato* is sensitive to red light of about 630 nm which acts as a releaser of innate courting behaviour (Crane 1955)

By using extracellular electrodes (Ag AgCl) the sensitivity to monochromatic light was tested between 310 and 650 nm on three *Heliconius* species *H erato H numata* and *H sara* All three species were found to be sensitive to all wavelengths tested with three sensitivity maxima at 370 and 470 nm for all of them at 550 in *H sara* and at 570 nm in *H erato* and *H numata* The eye of *H sara* showed

maximal sensitivity at 370 nm The sensitivity between 600 and 650 nm was found to be particularly good in *H erato* The well separated sensitivity maxima indicate the existence of at least two — possibly three — photopigments

The interspecies variation in spectral sensitivity demonstrated may be due to different spectral absorptive properties of screening pigments In microspectrophotometric measurements however the screening pigments in all three species have been found to be remarkably neutral (Langer and Struwe to be published)

It should be added that the specialization of the dioptric apparatus in *H erato* which causes a peak in the spectral sensitivity at 630 nm (Bernhard *et al* 1970) cannot account for the interspecies variation found in the present study

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C 27

Skin Circulation in the Cheeks of Arctic Populations and Adaptation to Cold

By M Wika *Institute of Zoophysiology University of Oslo, Norway*

Peripheral circulation in man has been mainly studied in the extremities Measurements of skin blood flow in other parts of the body are rare This study is part of a greater investigation on peripheral circulation and cold adaptation Cold adaptation can be of three types anatomical functional and psychological These adaptations can have their basis in adjustment of phylogenetic or ontogenetic nature I have tried to approach the problem of circulatory adjustment to cold stress with a new method and by investigating a part of the body which has not been paid much attention in this field of physiology namely certain areas of the face The cheeks have always been exposed to the cold environment in Arctic populations and should therefore be a part of the body which should be expected to be adjusted to cold

Lumberjacks in Southeastern Norway Skolt Lapps in Enare Northern Finland and Eskimos in Igloolik Nothern Canada are people that during their normal activity are exposed to cold Indoor workers from Oslo serve as controls Skin circulation is measured by the Xenon 133 clearance method developed by Sejrsen (1967, 1968) Before measurement the subjects are heated to a standardized state of heat dissipation and peripheral vasodilatation by enclosure to the neck in a styrofoam box

The Arctic populations the Skolt Lapps and the Eskimos have a lower circulation than the controls The Norwegian lumberjacks however seem to have a higher circulation than the controls

This study has been done to compare maximal capacity of skin circulation in Arctic populations with people living in warmer climate Populations that have lived

for generations and also during ontogeny in conditions of Arctic cold stress do not seem to have a higher vascularization in the skin of their cheeks than the controls It has been known for a long time that people in the Arctic seem to withstand cold better than ethnic groups in the South The conclusion from these results must therefore be that the seemingly higher resistance to cold in Arctic people if present must be due to functional or psychological factors

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From the Institute of Psychology, University of Bergen Norway

Spatial Specificity of Local Habituation to Cold

By

R Eide

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Abstract

Eide R *Spatial specificity of local habituation to cold* Acta physiol scand 1971 *82* 433—438

A series of daily cold exposures of 9 cm of the tail of 6 rats for 50 min for 17 days resulted in a

[illegible]

hour a day for 17 days A reduced cardio-acceleration was found Stimulation of the dorsal part of the same segmental area resulted in renewed cardio-acceleration This seems to indicate some degree of somatotopic organization of the habituation process

Severe local cooling of a part of the body has been found to result in an increase of blood pressure and heart rate in man (Hines and Brown 1932) as well as in species of animals such as the rat (Glaser and Griffin 1962) The nature of these reactions are not fully understood but they seem to be related to the pain elicited by the cold stimulus (Wolf and Hardy 1941 Wolff 1951 Whittow 1958)

On repeated exposures of the same body part to intense cooling a decline is found in the blood pressure reaction and the cardio acceleration This reduction in reaction has been termed habituation and evidence has been given that it is a central nervous process (Glaser and Whittow 1957 Glaser Hall and Whittow 1959 Glaser and Griffin 1962) although specific peripheral changes cannot be totally excluded

Glaser and Whittow (1957 have found that habituation to cold of one hand did not change the reaction to cooling of the other thus demonstrating a unilateral specificity of habituation and relating it to the unilateral representation of cutaneous hand sensation in the brain This principle was confirmed by Eagan (1961)

The questions raised in the present investigation were

1 Will the principle of spatial specificity of habituation hold also for an organ with bilateral representation in the brain?

2 Will there be spatial specificity of habituation also within a given dermatomic area?

Knowledge on these points might be helpful in the understanding of the central nervous mechanisms of the habituation process

To investigate this 2 different experimental series were carried out stimulating the tail of laboratory rats to various extents Heart rate was used as response variable

Experimental series I

A Materials

Male albino laboratory rats were used Group A consisted of 6 rats aged about 12 months, Group B of 4 rats aged about 16 months and the Control group of 8 rats aged about 18 months Their weight varied from about 300 to 500 g

The rats were placed in individual test boxes of such size that they could lie comfortably in a horizontal position but could not move freely or turn round The tail protruded through a hole at the end of the box

Stirred ice water in a thermos flask with temperature close to 0° C was used as cold stimulus The rat s tail was bent so as to pass through a hole at the top of the thermos flask and into the ice water

For recording of the heart rate 3 platinum electrodes were permanently inserted under the skin on the rat s back to which leads from an Elektromed mingograph 12 B were connected by small clips Temperature of the test room was 27° ± 1° C

B Experimental procedure

For all groups the cold experiments were preceded by a period of habituation to the experimental situation without cold exposure This was continued until the resting heart rate was stabilized which involved 1 to 2 weeks of daily exposure to the test box

On the test days the rats rested in the box until the heart rate was stabilized (approximately 30 min) The tail was then immersed in ice water to the desired level and the heart rate was recorded during the last quarter of each minute for the first 10 min of the exposure

The rats were given one continuous exposure daily of 9 cm of the tail Group A 6 rats) exposed for 50 min daily for 17 days Group B (4 rats) was exposed 30 min daily for 40 At the end of the habituation series an additional 3 cm of the tail was exposed

The rats in the Control group were exposed only twice on two different days once with 9 cm [illegible] the tail immersed and once with 12 cm Half of the group had the longer immersion first the other half the shorter

Results

The first exposure of 9 cm of the tail to ice water gave a mean increase in heart rate during 10 minutes of 63 beats/min in Group A On the 17th exposure this increase was reduced to 15 beats/min (Table I) According to the t test this decrease in reaction is statistically significant at the 0 05 level (Critical Ratio = 2 87)

On the 18th exposure an additional 3 cm of the tail was exposed to the cold so that 12 cm of the tail was immersed in ice water This gave a mean cardio-acceleration of 41 beats/min The mean difference in heart rate increase between the 17th exposure of 9 cm of the tail and the first exposure of 12 cm is 26 beats/min (Table 1) This difference is significant at the 0 01 level (CR = 8 24) Table I also shows that there are no great changes in the resting heart rate prior to the different exposures

The results from Group B are given in Fig 1 It will be seen that the tendency is exactly the same in this group There is a great variability of reactions (although all rats changed in the same direction) and the differences fall slightly short of statistical significance

TABLE I Heart rate response (beats/min) to ice water exposure Effect of habituation and spatial specificity (6 rats mean values and SD)

Exposure	Heart rate before exposure	Heart rate increase during ice water exposure
no 1 9 cm of tail exp	325±7 2	63±33 5
no 17 9 cm of tail exp	330±4 0	15±18 7
no 18 12 cm of tail exp	324±5.2	41±22 7

To test the possibility that the increased cardio-acceleration found on the exposure of an additional part of the tail could be the effect of spatial stimulus summation a Control group of 8 rats was tested on immersions of 9 cm and of 12 cm of the tail The mean heart rate increase upon immersion of 9 cm of the tail is 69 beats/min, and upon immersion of 12 cm 61 beats/min (Table II) There is thus no indication that spatial stimulus summation plays any part in the present experiments

TABLE II Effect of spatial stimulus summation Heart rate response to ice water exposure (8 rats, mean vaues and SD)

Part of tail exposed	*Heart rate before exposure*	*Heart rate increase during ice water exposure*
Distal 9 cm	376±15 3	69±48 2
Distal 12 cm	348±17 0	61±20 6

Experimental series II

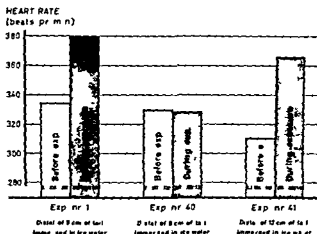
The results of Experiment I naturally led to the question if spatial specificity of habituation would also apply to stimulation within the same segmental area A second series of experiments were carried out to test this possibility

A Materials

8 male albino laboratory rats were used To obtain localized stimulation of the tail a brass tube was made 3 cm broad with flat surface into which was inserted transverse half cylindric furrows fitting around half the circumference of the tail Circulating ice water in the tube gave a surface temperature about 3° C In this way it was possible to stimulate separately either the ventral or dorsal part of a given 3 cm segment of the tail Skin temperature of the tail was measured by copper/constantan thermocouples connected to a potentiometer recorder with ice water reference

B Procedure

Prior to actual testing the animals were habituated to the experimental situation as in Exp I



C Results

There was found a mean reduction in cardio acceleration from the first to the 17th ventral tail exposure of 39 6 beats/min which is significant at the 01 level The dorsal exposure on the 18th day increased the cardio-acceleration with 25 4 beats compared to the 17th exposure This is also significant at the 01 level (Table III)

The minimum tail surface temperatures (on the opposite side of cooling) during exposure did not change significantly during the series (Table 4)

TABLE III Heart rate responses to tail cooling Effect of habituation and spatial specificity (8 rats mean values and SD

Exposure	Heart rate at rest	Heart rate increase
no 1 ventral	342 ± 11 2	48 12 0
no 17 ventral	316 ± 11 3	9 14 4
no 18 dorsal	318 8 6	34 5 8

TABLE IV Minimum tail surface temperature in C at different stages of the cooling series (8 rats mean values and SD

Exposure	Tail temperature
no 1 ventral	10 8 2 69
no 17 ventral	11 7 3 73
no 18 dorsal	12 3 1 73

The product moment correlation between tail temperature and heart rate increase (in exposure no 1 and no 17) was 20, which is not significant

Discussion

The present experiments show that there is a reduction in the cardio acceleration to exposure of the tail to ice water as a consequence of repeated exposures over several days This confirms earlier findings on man (Glaser and Whittow 1957, Glaser, Hall and Whittow 1959) and animals (Glaser and Griffin 1962) It is not likely that the reduced reactions should be due to the slight and insignificant changes in tail surface temperature or other peripheral changes Evidence for central nervous habituation to local cold stimulation has been given by Glaser and Griffin (1962), who found no evidence of local tissue changes as the basis of reduced reactivity in similar experiments, involving stimulation of rats' tail and gave evidence that the frontal lobes were involved Hernandez Peon and Brust Carmona (1961) have shown the brain stem to be involved in tactile habituation in cats. The importance of various central nervous structures in habituation to other types of stimulation has been established by Konorski (1948), Bard and Macht (1958), Thompson and Welker (1963) Butler and Harlow (1954) It is likely therefore that the reduced reactions here observed are due to habituation involving central nervous changes, although the evidence is not conclusive

Glaser and Whittow (1957) found that cold habituation in one hand did not affect the reactions to cooling of the other, and related this specificity to the unilateral representation of the cutaneous senses in the brain

The present investigation shows that habituation is specific also within a single organ with bilateral representation in the brain Habituation to tactile stimulation seems further to be specific also within a given dermatomic area (Experiment II)

This seems to indicate some degree of somatotopic organization in the central nervous structures involved in local cold habituation

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From the Department of Physiology, University of Goteborg Sweden

Aspects of the Cardiovascular Nervous Control in a Mammalian Diver (Myocastor Coypus)

By

B Folkow, B Lisander and B Öberg

Received 21 December 1970

Abstract

Folkow, B, B Lisander and B Öberg *Aspects of the cardiovascular nervous control in a mammalian diver (Myocastor Coypus)* Acta physiol scand 1971 82 439—446

Experiments were performed to analyse to what extent diving mammals represented by the

during a dive and hence distribute available oxygen reserves by means of a greatly reduced cardiac output preferentially to the brain and myocardium. Further, the vagal nerves exert on the heart ventricles a negative inotropic effect besides the profound negative chronotropic effect helping to keep cardiac output very low during submersion — The cardiovascular nervous control in the coypu is therefore in important respects more related to that in diving birds than to that in non-diving mammals which probably is a prerequisite for the endurance of prolonged submersion

Cardiovascular adjustments in diving animals during submersion have been widely studied above all in the duck and the general pattern of response is fairly well elucidated (*e g* Andersen 1966) It consists of a profound bradycardia and a corresponding decrease of cardiac output while systemic arterial pressure is well maintained indicating a strong peripheral vasoconstriction. This vasoconstriction takes place in skeletal muscles, viscera kidneys and in most of the skin and it may sometimes be so marked as to cause an almost complete closure of these major vascular beds The greatly reduced cardiac output thus seems to be diverted mainly to the myocardium and the brain so that the diving animal can virtually be considered as a heart lung brain preparation.

These spectacular adjustments in diving animals during submersion must imply quantitatively unique features particularly with respect to circulatory dimensions and

cardiovascular nervous control. Some aspects on these problems have been analysed on the duck (Feigl and Folkow 1963, Folkow, Fuxe and Sonnenschein 1966, Folkow and Yonce 1967, Folkow, Nilsson and Yonce 1967) and recently summarized (Folkow 1968). Vascularization of the skeletal muscles is thus more abundant in the duck than in non diving animals, while the larger arterial supply routes are more narrow and therefore of greater importance for regional flow resistance. The vasoconstrictor fibre influence on the muscle vascular bed is extremely powerful in divers and can virtually stop the blood flow, also during exercise. This is in contrast to the situation in *e.g.* the cat, where even intense vasoconstrictor fibre activation only phasically interferes with exercise hyperemia. Apart from a more abundant vasoconstrictor fibre supply in divers the explanation for this discrepancy seems to be differences in fibre distribution to the various consecutive vascular sections. Thus, in the duck an intense vasoconstriction occurs also in the larger arteries, which are situated outside the working mucles, and hence out of reach for vasodilator metabolites, implying that an intense constriction is here well maintained.

With regard to the neurogenic alterations of heart activity during diving, the pronounced vagal bradycardia is well known. However, besides this profound negative chronotropic effect of vagal discharge there is, in the duck, also evidence of a negative inotropic effect (Folkow and Younce 1967). For example, vagal activation of such intensities that occur during diving, causing at least a tenfold reduction of heart rate, leads to a 30—40 per cent reduction of stroke volume in the artificially paced ventricles despite a concomitant ventricular enddiastolic pressure rise.

In order to explore whether the mentioned characteristics of cardiovascular nervous control in ducks are valid also for diving mammals, and to explore some aspects that for technical reasons cannot be studied in ducks, a series of experiments was performed on a South American diving rodent *Myocastor Coypus* (the coypu or nutria) which displays a prompt and profound bradycardia when submerged.

Methods

14 expts. were performed on animals anesthetized with nembutal (50 mg/kg b.w.) or chloralose (30 mg/kg) given into a jugular vein which was exposed under light ether anesthesia. A tracheal cannula was inserted. Bloow pressure was recorded from the left carotid artery on a smoked drum with a mercury manometer or on a Grass Polygraph recorder via a Statham pressure transducer (P23AC). Blood flow was measured either in a skeletal muscle region (hind leg with the paw circulation excluded, 4 animals), in the kidney (4 animals) or in a suitable part of the small intestine (2 animals). After heparine (5 mg/kg b.w.) the draining vein was cannulated and the venous effluent was passed though an optical drop recorder, writing on a smoked drum or on the Grass Polygraph. The regional vasoconstrictor fibres, *i.e.* the lumbar sympathetic chain or the postganglionic sympathetic fibres running along the renal and the superior mesenteric artery, respectively, were carefully freed, cut centrally and placed on bipolar silver electrodes for stimulation. Supramaximal stimuli of graded frequencies were delivered from a Grass stimulator, model S4.

Cardiac output was determined repeatedly in 4 animals, using a thermodilution technique (Fegler 1954, Korner 1965). Via the brachial vein a thin bore catheter was located with its tip close to the right atrium so that small slugs of saline (0.5 ml) at room temperature could be injected. A small thermistor was advanced via one brachial artery to the aortic root for measurements of changes in aortic blood temperature. The thermodilution curves were recorded on the Grass Polygraph and extrapolated after plotting on a semilogarithmic paper. The area under the curve was determined by cutting it out and weighing it on a sensitive balanee.

… nded in two of these

introduced via the right carotid artery The pulse rate was recorded with a tachograph triggered by the upstroke of the ventricular pressure curve Cardiac output measurements were performed before during and after the stimulations utilizing the thermodilution technique

Circulatory adjustments to submersion were explored in three unanesthetized animals — About 2 weeks before the acute experiment a thoracotomy was performed and an electromagnetic flow probe (Micron Instruments Inc Model MQ-4050) was positioned around the ascending aorta. The leads from the probe were drawn out through the skin in the back of the neck and fixed with sutures The thorax was carefully closed during a transient hyperinflation of the lungs to reduce the extent of pneumothorax — The day before the acute experiment one carotid artery was exposed under ether anesthesia and cannulated with a heparinized catheter for blood pressure recordings (2 animals) During the experiments the arterial catheter was connected to a Statham pressure transducer (P23 AC) and the flow probe leads to a … Grass Polygraph … the upstroke … placing the … mals usually

Results

1 *Experiments on anesthetized animals*

Cardiac output was determined in 4 anesthetized animals, the average value being 158 ± 15 ml/min × kg b w

Fig 1 upper panel shows the effects of supramaximal vasoconstrictor fibre stimulation at different frequencies on calf muscle blood flow Even very low frequencies (one impulse every other second) induce a clearcut vasoconstriction At 2 imp/sec resistance increases by approximately 700 per cent and flow virtually stops already at 6 imp/sec In contrast maximal constriction occurs first around 10—12 imp/sec in the cat and flow resistance is then increased only some 5—700 per cent

The muscle vasoconstrictor response in the coypu was abolished but never reversed to a vasodilatation after administration of dibenzyline 2—3 mg/kg b w (2 animals) speaking against the existence of any significant vasodilator fibre supply to the muscle vessels in this species However resting blood flow in the acutely denervated calf muscles of the coypu was fairly high and even maximal exercise or i.a acetylcholine injection (5 μg) did not increase flow more than 2—3 times at most This indicates a low basal tone in the muscle vessels in the coypu compared with the situation in e g cats and dogs A mere inhibition of constrictor fibre tone in the coypu is therefore in itself capable of increasing muscle blood flow to almost the same extent as an activation of the cholinergic dilator fibres in the mentioned non-diving species

Very powerful vasoconstrictor responses to sympathetic stimulation were seen also in the kidney of the coypu (Fig 1 lower panel) However the lowest frequencies (below 3—4 imp/sec) produce comparatively moderate resistance increase, but impulse rates above this level can virtually stop renal blood flow The frequency response curve thus displays a very steep rise in the frequency range between 3—4 imp sec,

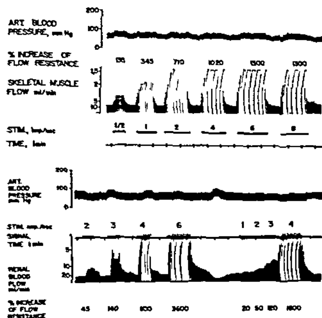


Fig 1 Effects of vasoconstrictor fibre stimulation at different frequencies on blood flow in the calf muscle (upper panel) and the kidney (lower panel) in the anesthetized coypu

in which range renal flow resistance increases some 10—20 times — Cats in comparison show far weaker neurogenic vasoconstriction in the kidneys (*e g* Celander 1954)

Very intense neurogenic constrictions were observed also in the intestinal vascular bed of the coypu where a 5 fold increase of resistance was obtained at stimulation frequencies of 2—4 imp/sec a 10—15 fold resistance increase at 4—6 imp/sec, and 25—30 fold increase of flow resistance at 6—8 imp/sec Moreover in contrast to the situation in *e g* the cat intestinal blood flow was maintained markedly reduced throughout the whole period of stimulation There was thus no sign of any autoregulatory escape *i e* a more or less complete recovery of blood flow towards control values despite continued activation of the vasoconstrictor fibres which is so typical for the gastrointestinal vessels of cats (Folkow *et al* 1964) It follows that the coypu by means of a moderate vasoconstrictor fibre activation can keep intestinal blood flow effectively reduced to very low levels during *e g* a sustained dive again a necessity for directing the available oxygen reserves preferentially to the brain and heart.

In Fig 2 the neuro-effector characteristics in the mentioned three vascular beds of the coypu are presented as frequency response curves Corresponding data for the skeletal muscle of the cat (Mellander 1960 are shown for comparison The figure shows that the curve for the muscle vessels is markedly shifted to the left in the diving species as compared to the non-diver The intestinal and renal response curves in the coypu show a similar shift to the left as compared to the respective response curves in the cat (Celander 1954 not shown in Fig 2) This implies that far more intense

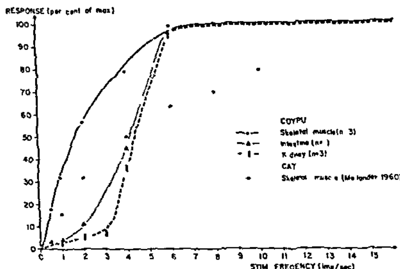


Fig 2 Relation between stimulation frequency and flow resistance expressed as per cent of the maxiaml response in skeletal muscle intestine and kidney of the coypu and in skeletal muscle of the cat (from Mellander 1960) The curves from the coypu experiments are means of 2—4 stimulations with each frequency in *n* number of animals

maximal vascular responses are reached at decidedly lower discharge rates in the coypu than in the non diver

Fig 3 shows the effects of vagal stimulation (10 imp/sec) on left ventricular pressure when heart rate is kept constant by ventricular pacing In the unpaced heart such a stimulation frequency produces a comparable intense bradycardia as that occurring when the unanesthetized animal is submerged It is seen that a substantial reduction of end systolic pressure is induced combined with a moderate rise of end diastolic pressure The pressures are promptly restored to control upon cessation of stimulation During vagal stimulation of the paced heart cardiac output fell by approximately 25 per cent implying a corresponding reduction of stroke volume,

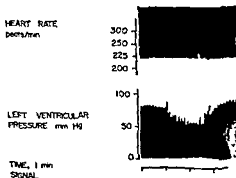


Fig 3 Effects of efferent vagal stimulation (8 V 3 msec 10 imp/sec) on left ventricular pressure in the artificially paced heart Note the increase of end-diastolic pressure

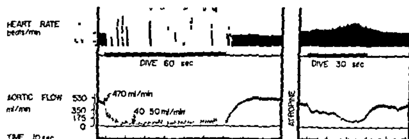


Fig 4 Effects of submersion of the unanesthetized coypu on heart rate and aortic flow before (left panel) and after administration of atropine (right panel)

despite the raised end-diastolic pressure This strongly suggests a negative inotropic effect on the ventricular myocardium Atropine, 0 5 mg/kg completely abolished these effects

2 Experiments on unanesthetized animals

3 unanesthetized animals were submerged while blood pressure, heart rate and aortic blood flow were continuously followed — Control aortic blood flow averaged 175 ml/min × kg b w In case roughly 5 per cent of cardiac output (C O) is distributed to the coronary vessels, the mentioned figure corresponds to a C O of approximately 185 ml/min × kg b w , i e a value 10—15 per cent higher than in the anesthetized coypu — Fig 4 shows the effect of one minute of submersion An almost instantaneous bradycardia reducing heart rate from 210 to 30—35 beats per min occurs together with an almost as immediate, about 10 fold reduction of aortic blood flow Arterial blood pressure remained essentially constant (not shown in the figure) This indicates an intense and prompt rise of systemic flow resistance reaching a roughly tenfold increase within 10—15 sec These figures together with the experiments on the anesthetized coypu suggest that the neurogenic vasoconstriction must be so intense during submersion as to nearly stop blood supply in skeletal muscle kidneys and gastrointestinal tract, and therefore the greatly reduced cardiac output is preferentially diverted to the brain and the myocardium — After atropine (0 5 mg/kg b w) submersion induced only a minor reduction of heart rate and this cardiac slowing developed more slowly during the dive Evidently the prompt and intense slowing of the heart normally seen during a dive is mainly of vagal origin, the remaining slight bradycardia might reflect an inhibition of discharge in the sympathetic accelerans nerve (cf Folkow and Yonce 1967)

Discussion

The present experiments were undertaken mainly to establish whether some of the characteristics of cardiovascular nervous control in diving birds, as in the duck (Folkow, Fuxe and Sonnenschein 1966 Folkow Nilsson and Yonce 1967 Folkow and Yonce 1967, Folkow 1968 are present also in diving mammals In the present

study, a South American rodent the coypu or nutria was used as a representative for mammalian divers Unfortunately this species proved to be otherwise rather unsuitable for investigations that call for more extensive surgical interventions partly because of their suceptibility to anesthesia partly because the great fraglity of some tissues, particularly the blood vessels which often led to massive bleedings despite cautious preparation However from the technically successfull experiments it seems clear that the coypu with regard to the neurogenic cardiovascular control is more closely related to diving birds than to non diving mammals such as cats and dogs The marked and prompt bradycardia and reduction of cardiac output during sub*mersion is thus found in virtually all diving vertebrates* (Andersson 1966) but only in such species When *e g* a cat is submerged the bradycardia is moderate and develops gradually and cardiac output is only slightly reduced (Lisander unpublished observations)

The vasoconstrictor fibre influence on the vascular beds of skeletal muscle kidney and intestine is far more pronounced in the coypu than in the cat but appears to resemble quantitatively that of the duck to judge from the profound increases in *flow resistance occurring in this species during submersion* (Folkow Nilsson and Yonce 1966) It is reasonable to suppose that this very intense vasoconstriction *e g* in the skeletal muscles involves also the larger precapillary resistance vessels as is the case in the duck (Folkow Fuxe and Sonnenschein 1966) and evidently also in the seal (Irving Scholander and Grinnell 1942) Counteracting vasodilator metabolites cannot here interfere with the intense neurogenic vasoconstriction Whatever the background for the very pronounced vasoconstrictor responses might be fairly *moderate discharge rates in the vasoconstrictor fibres can virtually stop the blood flow* through the mentioned circuits in the coypu and therefore divert the cardiac output mainly to the brain and heart during a dive

The experiments with paced hearts indicate that the coypu displays a vagal negative inotropic effect on the ventricles in the coypu as in the duck Folkow and Yonce 1967) This agrees well with the findings of Ferrante Browner and Opdyke (1968) who report a 25—30 per cent decrease in left ventricular contractility when the unanesthetized coypu was submerged It has in fact been reported that a negative inotropic effect of vagal origin can be traced even in non-diving animals as in the dog (*e g* De Geest *et al* 1965) while other workers have found no evidence of such an effect (*e g* Linden 1963) Both in the duck and the coypu however this vagal negative inotropic influence is considerable at least at such vagal discharge rates which appear to occur during submersion and are then capable to reduce heart rate some 10 times In such a way the heart of the diving species can maintain a low stroke volume in connection with a very low heart rate, despite an increased diastolic filling of the ventricles and thus counteract the Frank Starling forces operating in such circumstances. The profund bradycardia in comb nation with the considerable restraint on ventricular contractility must imply a marked suppression of myocardial *oxygen needs during submersion*

Thus with respect to the nervous control of the circulation there seems to be

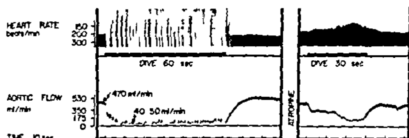


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greater similarities in many respects between diving mammals and diving birds than between diving and non diving mammals The described peculiarities in diving species, with regard to the neuroeffector organisation in the cardiovascular system are no doubt one of the more important prerequisites for the endurance of prolonged sub mersion since they are all important for the saving of the limited oxygen reserves in blood and lungs for preferential delivery to the heart and brain One should how ever hardly consider the mentioned characteristics of circulatory control in diving species as qualitatively unique they may rather be looked upon as extreme reinforcements of control devices that exist in all vertebrates

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From the Physiology Section Johan Throne Holst s Institute for Nutrition Research University of Oslo Norway

A Fifth Modality of Taste

By

H T ANDERSEN and A O HARTMANN

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Abstract

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An analytical search for independent modalities of taste is presented The correlation matrix between independent stimuli was calculated using data recorded from chorda tympani single fibres in rats (Erickson Doetsch and Marchall 1965) assuming that specific taste sensations may be explained by an across fibre pattern input into gustatory primary afferents The four main factors were first rotated since this number of factors would correspond to the common idea of four independent taste primaries bitter, salty sour and sweet The corresponding re solution of stimuli on four factors was inadequate However by adding one more factor a striking separation on five factors was obtained The salts used separated distinctly on two of the factors the lithium and sodium salts on one and the salts of the larger cations on the other Sucrose HCl and quinine hydrochloride correlate heavily with one and only one of the three remaining factors These five main factors account for 92 6 % of the variance in the material The conclusoin may be drawn that an across fibre pattern contains taste moduli con sistent with the established concept of taste primaries the primary referred to as salty however seems to consist of two independent moduli

Studies of taste specificity mechanisms commonly imply the existence of four fun damental gustatory qualities *i e* bitter salt sour and sweet Thus electrophysiological investigations are usually carried out by analyzing the response of single fibres in gustatory primary afferents upon stimulation of the receptor organ by sapid solutions tasting bitter salty sour or sweet to the research worker The substances most fre quently employed in such experiments include quinine hydrochloride sodium chlo ride hydrochloric or acetic acid and sucrose respectively and it is tacitly understood that each of these stimuli affect one and only one receptor type

The concept of four basic taste qualities may or may not be justifiable However the idea has prevailed ever since Fick (1864) first identified bitter salty sour and sweet as gustatory primaries In fact von Bekesy (1964 1966) who studied the pos sibility of detecting any number other than just four fundamental taste modalities in humans concluded that man possesses only the four gustatory qualities recognized by Fick

There are several reasons for leaving the question of additional taste modalities open. Unsuccessful attempts of finding new gustatory qualities do not exclude their possible existence. Moreover, any monogustatory sensation that man assigns to a given gustatory stimulus depends not only on the chemical compound used but on its concentration in the test solution as well. This phenomenon may apply equally to the gustatory apparatus of other species. Furthermore, identification and grouping of taste sensations into four basic gustatory qualities may be a consequence of established semantical usage (for a discussion see Andersen 1970).

The purpose of the study reported here was to investigate the possibility of *identifying specific taste modalities by applying factor analysis technique to a suitable* set of data.

Material and Methods

The data obtained by Erickson, Doetsch and Marshall (1965) was selected for analysis. Their report presents the electrical response recorded from 62 single neural elements in the chorda tympani nerve upon application of various test solutions to the tongue. Moreover, Erickson *et al* (1965) include a correlation matrix for each pair of the 13 different chemical compounds used as stimuli (*cf* Table II and III). Their material was obtained from 44 female Sprague-Dawley rats.

Factor analysis was performed by determination of principal factors which were subsequently rotated using the varimax method as described by Harman (1967).

The correlation matrix of Erickson *et al* (1965) has served as a basis for the present study assuming that the "across fibre pattern" hypothesis of Pfaffmann (1941, 1955) and Erickson (1963) may explain the taste sensations evoked by activity in peripheral gustatory nerves.

The taste stimulus (Z_i) in our model is assumed to consist of linear combinations of elements contributed from independent taste qualities (G_j), here called factors. Thus

$$Z_i = \sum_{j=1}^{m} a_{ij} G_j \qquad (1) \qquad i = 1, 2, 3, \ldots n$$

or in matrix notation

$$\mathbf{Z} = \mathbf{AG} \qquad (2)$$

The number a_{ij} is usually referred to as the factor loading of the ith variable on factor j. The correlation matrixes for the variables (the taste stimuli) is then

$$\mathbf{R} = \mathbf{AA'} \qquad (3)$$

except that the diagonal elements will contain the communalities instead of unity. The communality for each variable is defined by

$$C_i = \sum_{j=1}^{m} a_{ij}^2 \qquad (4)$$

The communality tells how much of the variance for each variable which is accounted for by the actual factors. The total contribution from the factor G_j to the variance is defined by

$$V_j = \sum_{i=1}^{n} a_{ij}^2 \qquad (5)$$

The method of finding principal factors starts with the conditions of first maximizing V_1, then V_2 and so on. This leads to an eigenvalue problem

$$\mathbf{Q'RQ} = \mathbf{\Lambda} \qquad (6)$$

where $\mathbf{Q}$ is the orthogonal matrix reducing the symmetric correlation matrix $\mathbf{R}$ to a diagonal matrix $\mathbf{\Lambda}$. The diagonal elements in $\mathbf{\Lambda}$ are the eigenvalues, the columns of $\mathbf{Q}$ are the eigenvectors. The solution gives the matrixes $\mathbf{Q}$ and $\mathbf{\Lambda}$, and the a_{ij}'s for a factor G_j can be derived from the eigenvector number j by a scaling using the eigenvalue λ_j.

Thus we have determined the matrix A which gives the loadings on the factors G The orientation of these factors in the space, however, is not definitive Any orthogonal transformation of these factors will be an adequate solution as represented by

$$F = TA \quad (7)$$

T is then a matrix for an orthogonal rotation of the factors G F is the factor scores on the new factors H, and the stimuli may equally well be expressed in this new factor space

$$Z = FH = AG \quad (8)$$

The transformation matrix T may be computed in various ways We have used the varimax criterion (Kaiser 1958) which usually gives relatively specific and simple factors as a resul of rotation

Different numbers of the most important factors (G_J) may therefore be rotated to study any possible latent characteristics pertinent to our problem

The analytical procedures were performed at the Computing Centre at the University of Oslo using a standard program on the CDC 3300 computer

Results

The 6 first factors obtained by factorization[1] using 6 factors are presented in Table I Although most of the variance may be explained by a smaller number of factors than the 6 first it does not seem justified to choose 4, because less than 90 per cent of the variance is accounted for by the first 4 factors Nevertheless, it is important to show the varimax rotation of 4 factors since this would conform to the idea of 4 gustatory primaries

Rotation of 4 factors

The results obtained by rotating 4 factors are shown in Table II It appears that the first two factors correlate heavily with the salts The first factor brings out the lithium and the sodium salts whereas the second factor is associated with the salts of the larger cations Moreover, it should be noted that hardly any analytical difference exists between sodium hydroxide and sodium chloride Sucrose emerges with the third factor while hydrochloric acid mainly appears on the fourth However quinine hydrochloride is to some extent correlated with three different factors the second the third and the fourth

TABLE I The 6 first factors in the data of Erickson *et al* (1965

No of Factor	Eigenvalue	Percent of variance	Cummulative Percent
1	5 48	42 2	42 2
2	4 16	32 0	74 2
3	1 04	8 0	82 2
4	0 84	6 4	88 6
5	0 51	3 9	92 6
6	0 37	2 8	95 4

[1] The communalities in the diagonal of the correlation matrix were estimated to be unity

TABLE II Varimax rotation of 4 factors

(j)	Variable	Communality (C_j)	Factor loadings (F_{ij}) 1	2	3	4
Factor no (i)			1	2	3	4
% of total variance			38 3	29 5	9 1	11 7
1	NaCl	94	92	04	— 03	30
2	Na_2SO_4	92	93	— 19	04	— 12
3	$NaNO_3$	91	90	14	26	— 01
4	LiCl	91	90	03	05	30
5	Li_2SO_4	72	84	— 12	09	01
6	NaOH	81	89	13	11	01
7	KCl	86	— 12	90	03	20
8	NH_4Cl	92	— 03	92	— 10	25
9	$MgCl_2$	99	24	92	09	30
10	$CaCl_2$	95	— 02	97	06	01
11	HCl	89	11	34	— 02	87
12	Quinine	77	15	44	42	62
13	Sucrose	93	20	00	94	06

Rotation of 5 factors

Separation into 4 fundamental taste qualities is by no means evident in our rotation of four factors In fact the logical consequence of the results obtained is to suspect that two different salt modalities may exist (*cf* Table II) When the number of the factors rotated was increased to 5 the data shown in Table III emerged Here 2 salt groups sucrose hydrochloric acid and quinine hydrochloride separate distinctly in the 5 different factors

It will be seen that the communality of lithium sulphate is rather on the low side Rotation with 6 factors results in a more satisfactory communality for this salt which

TABLE III Varimax rotation of 5 factors

(j)	Variable	Communality C_j	Factor loadings (F_{ij}) 1	2	3	4	5
Factor no i			1	2	3	4	5
% of total variance			38 4	28 9	8 2	8 4	8 7
1	NaCl	94	92	03	— 05	24	— 16
2	Na_2SO_4	92	93	— 20	03	— 12	04
3	$NaNO_3$	91	90	12	21	— 12	— 17
4	LiCl	92	91	07	03	25	— 17
5	Li_2SO_4	73	84	— 12	10	02	02
6	NaOH	82	89	14	13	02	02
7	KCl	87	— 12	90	05	18	— 10
8	NH_4Cl	93	— 02	93	— 09	24	— 10
9	$MgCl_2$	1 00	24	88	— 03	08	— 47
10	CaCl	96	— 02	98	07	01	— 03
11	HCl	99	12	37	05	89	— 20
12	Quinine	1 00	14	37	20	23	— 87
13	Sucrose	99	21	00	96	04	— 13

to some extent separates on the sixth factor But even so, the correlation of lithium sulphate is higher with the first salt dimension (76 *vs* 59) A low correlation of 07 between this sixth factor and the other sulphate containing salt ($Na\ SO_4$) was found in the rotation

Discussion

The validity of the conclusions drawn from the work presented here is limited by the imperfect data available However, the results indicate that scientific inquiry into mechanisms of taste specificity should take into account the possible existence of a fifth gustatory dimension This new gustatory quality emerges by dividing the salts commonly used in taste experiments into two groups The one group seems to include the salts of lithium and sodium The second category contains salts with larger cations The anionic component of the various salts studied does not seem to contribute significantly to the taste quality elicited This latter finding support a conclusion already arrived at by others (see Pfaffmann 1961) Finally, the "alkaline" taste quality occasionally referred to did not separate as a distinct gustatory dimension in our data To the contrary sodium hydroxide and sodium chloride were not separated by the rotation

Previous attempts (Schiffman and Falkenberg 1968) to analyse the most important gustatory dimensions in the data of Erickson *et al* (1965) rested on the assumption that the raw data frequency recordings are describable by the product of 2 matrixes One of these matrixes emerges from row vectors identifying the neurons the other may be formed from a set of column vectors describing the stimuli used Schiffman and Falkenberg (1968) concluded that the number of rows and columns respectively, necessary to reproduce 98 per cent of the variance of the original data was three, two of which were labelled dimensions of taste quality However neither neurons nor stimuli showed any apparent clustering and the taste modalities commonly recognized did not prove independent of each other by this analytical treatment

The factor analysis procedure used in our investigation strongly supports the idea that there are specific and independent taste modalities This does not necessarily imply that the corresponding monogustatory stimuli exist It is concluded therefore that specific taste sensations may be explained by an across fibre pattern ' input in gustatory afferents

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From the Department of Physiology, Veterinärhögskolan, Stockholm, Sweden

On the Relation between the Chorda Tympani Nerve Response, Arterial Oxygen Tension and Blood Flow in the Tongue of the Rat

By

GORAN HELLEKANT

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Abstract

HELLEKANT, G *On the relation between the chorda tympani nerve response, arterial oxygen tension and blood flow in the tongue of the rat* Acta physiol scand 1971 82 453—459

The summated chorda tympani response of the rat to taste stimulation was recorded while, (a) the rat was artificially ventilated with hypoxic mixtures of oxygen nitrogen, (b) the blood flow through the ipsilateral common carotid artery and the intra arterial oxygen tension were [illegible]

changes of the blood flow to the tongue

A previous study (Hellekant 1971) shows that the taste response of the chorda tympani nerve in the rat is sensitive to changes of the blood flow through the tongue Thus the taste response disappeared within 5 min when the blood flow to the tongue was arrested An attempt to discuss this phenomenon was made in that study It was then suggested, chiefly on comparative evidence that inability to supply oxygen to the taste buds was probably the primary cause of the described decrease in the taste response However it seemed of interest to study more closely the physiological significance of the oxygen tension of the blood and blood flow through the tongue on the gustatory response in the rat The present investigation was made with this aim

Methods

[illegible] albino rats [illegible] anaesthesia [illegible], or a com- [illegible] were gi[illegible]

in a previous study (Hellekant 1971) Gallamine iodide was often administered as muscular relaxant when the animals were artif ... 1® or Dextran were used as

... cut centrally The response ... on, amplified and displayed, summated and recorded on a Statos I recorder (Varian assoc) The ... elsewhere (Andersson et a ... artery except during the e ... femoral artery was used T ... (Beckman 315780 Micro ... of the abdominal aorta ... with an electromagnetic flow meter (Statham M-4000) in the right common carotid artery, since the flow through the lingual artery was too small to measure with the instruments available

The tongue was unilaterally perfused In most cases the contralateral blood supply was not touched This seemed to be justified because each half of the tongue is largely supplied with blood from the ipsilateral lingual artery (Hellekant 1971) The effect of ... flow was always tested ... 2 ways stopcocks with an ... loop had a volume of 0 ... tongue or to a femoral v ...

... aggregates of platelets created a considerable problem They raised the vascular resistance of ...

... (Wistar albino rats 189 D, from Agricultural Research Councils Field Station at Compton England) which is less sensitive to Dextran and I was able to do these experiments with this strain, though the tendency of platelets to aggregate was not completely abolished

Results

Fig 1 shows the results of an attempt to study the effect of lowered oxygen tension of the inspired air on the taste response The animal was artificially ventilated The stroke volume and frequency of the respirator were constant The periods of ventilation with low oxygen content (the test periods) were interspaced with periods of ventilation with air (control periods) During the test periods different mixtures of oxygen in nitrogen (varying from 6 to 16 % O_2) were administered The test periods for these oxygen mixtures lasted about 6 min The summated chorda tympani response to Ringer's solution 0 5 M sucrose, glucose and NaCl was recorded after about 4 min, range 216 to 268 sec The test period for the 6 per cent oxygen mixture lasted 12 min, during which the series of stimuli was repeated twice, after 4 and 10 min respectively The control periods lasted for about 11 min The taste response to the stimuli mentioned was obtained after about 9 min, range 452 to 740 sec The average value of the response to each solution during the control periods before and after each test period was then calculated The ratio between this value and that obtained during the intervening test period was calculated and plotted (Fig 1) against the oxygen content in the test period in question The regression line

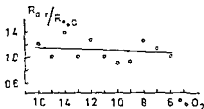


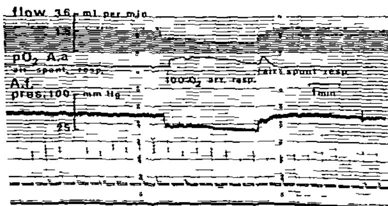
Fig 1 The diagram expresses as ratios the effect of

(R) obtained during the different periods were calculated and plotted against the oxygen content of the inspired gas mixtures The regression line is included

was calculated and drawn Fig 1 shows that it is almost parallel with the abscissa The correlation coefficient (0 29) does not significantly differ from zero This indicates no change of taste response with decreasing oxygen content of the inspired air Similar observations were obtained to the other stimuli tested It may be worth mentioning that the animal appeared cyanotic after ventilation with the 6 per cent oxygen mixture From this and other experiments it can be concluded that the animals were able to maintain their gustatory sensitivity until the hypoxia caused a failure of their circulation

It then seemed of interest to see whether a similar absence of effects on the taste response would be observed during increased oxygen tension but decreased blood flow to the tongue

Fig 2 represents an attempt to study this relation Fig 2 is composed of four simultaneous recordings, the pulsatile blood flow in the ipsilateral common carotid artery, the arterial oxygen tension measured in the abdominal aorta, the systemic blood pressure recorded in the femoral artery and the summated chorda tympani response to repeated rinses of 0 3 M NaCl over the tongue The first arrow in Fig 2



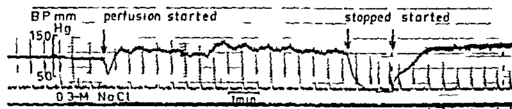


Fig. 3. The taste response to 0.3 M NaCl and the blood pressure near the branching of the lingual artery were recorded while the blood flow from the heart to the tongue was arrested and replaced by blood from an external perfusion loop. The record shows that this flow maintained the taste response.

indicates when the rat was switched from spontaneous respiration of air to artificial ventilation with pure oxygen. Fig. 2 shows that this caused a diminution of the cardiac output and a fall of the already low blood pressure. The recording also demonstrates that this elicited a decrease in the taste response. Artificial ventilation was then stopped at the second arrow. This increased cardiac output and systemic blood pressure. The record indicates that the oxygen tension rose briefly after ventilation with pure oxygen had ceased. This was probably caused by the faster flow of oxygen-rich blood around the oxygen electrode due to increased cardiac output, before less saturated blood reached the electrode.

It can be concluded that Fig. 2 demonstrates that despite a rise in blood oxygen tension a decrease in taste response can be recorded if the blood flow diminishes.

Fig. 3 illustrates further the effect of blood flow to the tongue on the taste response. The taste response to 0.5 M NaCl and the arterial blood pressure of the tongue were recorded simultaneously. Fig. 3 shows that the blood supply to the tongue ceased when the external carotid artery was occluded between the branching of the lingual artery and the heart. The perfusion pump was then started. It replaced the heart and supplied a blood flow which was adjusted to keep the blood pressure to the tongue at approximately the same level as before. Fig. 3 demonstrates that this blood flow maintained the taste sensitivity. It shows also that the taste response depended upon the flow because it deteriorated when the pump was stopped.

In Fig. 4 the tongue was perfused in a similar manner as described above. The taste response to 0.3 M NaCl and the perfusion pressure were recorded as in Fig. 3. They were then measured and plotted in Fig. 4. Fig. 4 shows that the response could be increased by the increase in blood flow to the tongue. The graph demonstrates that a 40 per cent increase of perfusion flow more than doubled the taste response in this case. In general a perfusion rate of about 0.3 ml/min kept the pressure of the lingual artery and taste response at the same level as before the occlusion.

In summary, the results of this study demonstrate that changes of the arterial oxygen tension did not affect the taste response as long as the circulation did not fail. Further they show that the taste response could be maintained and influenced through perfusion of the tongue.

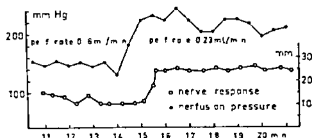


Fig 4 The diagram shows the effect of an increased perfusion rate to the tongue on the blood pressure of the lingual artery and on the summated nerve response to repeated stimulation with 0 3 M NaCl The record shows that an increase of flow increased the taste response

Discussion

In a previous study (Hellekant 1971) it was shown that the taste response disappeared if the blood flow to the tongue was arrested Deficiency of oxygen was then suggested as the primary cause The results of Fig 1 seem to contradict this suggestion There may be three explanations for this

1 First the interval between the onset of ventilation with the hypoxic gas mixtures and the recording of the taste response might have been too short, the 4 min period used may have been insufficient to lower the oxygen tension of the blood to a value proportional to the tension of the inspired air This is probably not true for two reasons First the study by Comroe *et al* (1962) shows that such a value can be expected to be reached within one min Second, the *in vivo* measurements of oxygen tension exemplified in Fig 2 indicate a time of less than one min to reach the new level It can therefore be concluded that the blood oxygen tension very likely reached a level proportional to the oxygen tension of the inspired gas mixture

2 It may be suggested that the oxygen storing capacity of the taste cell structure was large enough to compensate for the difference between the amounts of oxygen consumed and transported per unit time This suggestion cannot be entirely ruled out but it seems probable that some change would have been observed after 10 min of ventilation with 6 % O if this was the only explanation

3 The blood of the rat becomes saturated to about 50 % in 6 % O (Altman and Dittmer 1961 p 153) Although the value is low it indicates that the taste receptor cells could have been supplied with enough oxygen through an increase of the circulation This suggestion is further supported by the general observation during this series that the rats maintained their taste sensitivity until their hearts failed It is therefore felt that this is the most probable explanation

This first part of the discussion shows that lowering of the arterial oxygen tension did not solve the question of whether oxygen deficit or some other factor caused the decay of response described in an earlier study (Hellekant 1971) Instead it further emphasized the importance of circulation in taste sensitivity Accurate determination or control of blood flow to the tongue seemed necessary for a further analysis The flow meters available were not sensitive enough to measure blood flow through the lingual artery of rats Perfusion of the tongue seemed to offer one solution

Many factors have been found to contribute to progressive deterioration in

quality of perfusion (Norman 1968) I found that some of these factors could be avoided if the extra corporeal loop for the blood was made as short as possible Despite this, perfusion of the rat tongue met considerable difficulties These were not only caused by the low flow rates and the tiny vessels, aggregates of platelets were another problem

It may be worthwhile to describe briefly the effects of formation of such aggregates A steep rise of the vascular resistance of the tongue was the first sign recorded after aggregates were observed in the perfusion loop If perfusion was not stopped, the taste response then diminished and disappeared No attempt was made to study the effects of the aggregates on the response to mechanical and temperature stimulation but it is felt that the response to these stimuli was maintained for longer The observed increase of perfusion resistance indicates that these aggregates occluded the vessels and caused local ischaemia The effect of the ischaemia was first manifested on the taste response This indicates a higher metabolic rate of the taste receptor cells than of the other sensory end organs of the tongue

No attempt was made to quantify the relation between perfusion flow and taste response Unknown factors as well as uncontrollable ones contributed to this relation Differences in the electrical recording conditions individual variations in taste sensitivity, and anatomical differences in the area supplied by the lingual artery were among the factors which were observed but cannot be rated However it was found that a perfusion rate of 0 3 ml/min served as a good standard flow Provided no other factor varied it was possible to increase sustain and decrease the taste response by changes of the perfusion flow

It is well known that electrical stimulation of the peripheral part of the chorda tympani nerve increases the blood flow of the tongue (*e g* Erici and Uvnas 1951) This increase in flow has been regarded by several investigators as the result of activity in glandular structure (*cf* Gautvik 1970 a) However, recent studies by Gautvik (1970 a b) show that there are true vasodilatory fibres in the submandibular salivary gland of the cat This makes the existence of such fibres to the tongue more likely An earlier study (Hellekant 1970) demonstrated that chorda tympani stimulation in the rat decreased the vascular resistance of the tongue and gave an increased blood flow through the tongue with the same blood pressure The present study shows that changes in the blood flow through the tongue influence taste sensitivity On the basis of these observations it may be suggested that there may be a mechanism for a general modulation of the peripheral taste sensitivity in the rat

In summary this discussion indicates that oxygen deficit cannot be ruled out as a possible cause for the observed decline of taste sensitivity when circulation to the tongue ceases It stresses further the importance of blood flow the taste receptor cells and indicates that their response can be modulated by vascular changes in the tongue of the rat

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From the Department of Physiology I and the Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden

Antagonism of Slow Reacting Substance by Polyphloretin Phosphate on Isolated Human Bronchi

By

A. A. Mathé[1] and K. Strandberg

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Abstract

Mathé, A. A. and K. Strandberg. *Antagonism of slow reacting substance by polyphloretin phosphate on isolated human bronchi.* Acta physiol. scand. 1971. 82. 460—465.

Purified slow reacting substance (SRS) induced a marked and long lasting construction of isolated human bronchi. No signs of tachyphylaxis in the response to SRS were observed. [illegible] ergic receptors did [illegible] nd dibutyryl cyclic [illegible] phosphate (PPP) [illegible] isolated guinea pig ileum. Antagonism by PPP could be surmounted by higher doses of agonist and there was a parallel shift of the dose response curves of the agonist indicating a competitive antagonism. PPP did not modify the bronchoconstriction induced by acetylcholine and histamine.

Bronchoconstricting action of partially purified slow reacting substance (SRS) (Brocklehurst 1956, Berry and Collier 1964) and of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Änggård and Bergström 1963, Sweatman and Collier 1968) has been demonstrated and is of interest in view of the possible role in bronchial asthma of these two substances. SRS is released from lungs of asthmatic patients challenged *in vitro* with specific allergen (for references see Brocklehurst 1962) and from human lungs passively sensitized with reaginic serum (Parish 1967, Sheard, Killingback and Blair 1967). Furthermore, release of SRS (Kellaway and Trethewie 1940, Brocklehurst 1962) as well as of $PGF_{2\alpha}$ (Piper and Vane 1969) has been demonstrated in anaphylaxis of the guinea pig lung.

Polyphloretin phosphate (PPP), a polymer with a molecular weight of about 15,000 (Diczfalusy *et al.* 1953), antagonizes the action of prostaglandins on isolated organs of several animal species (Eakins, Karim and Miller 1970). Recently it was shown that PPP inhibits the constricting action of $PGF_{2\alpha}$ on isolated human bronchi (Mathé, Strandberg and Åström 1971). Since both $PGF_{2\alpha}$ and SRS (Strandberg and Uvnäs 1971) are unsaturated hydroxy-acids of lipid nature we decided to also test the effect of PPP on SRS induced bronchoconstriction.

[1] Permanent address: Psychophysiology Laboratory, Boston University School of Medicine, Boston, Mass.

Methods and Materials

Bronchi were obtained from macroscopically normal parts of human lungs which had been were cut following NaH_2PO_4 93.5 %

O_2+6.5 % CO_2. The strips were put under a tension of 0.5 g and the changes in tone measured isometrically with a Grass force-displacement transducer model FT 03 and recorded on a model 5 Grass polygraph. After suspension the preparation was allowed to equilibrate for at least 1 hr after which time the tension was adjusted to the original 0.5 g and the experiment started

measurable contraction under specified conditions of guinea pig ileum tested on 10 animals (Strandberg 1971)

Results

The mean concentration of SRS necessary to induce threshold bronchoconstriction tested on 6 bronchi was 2.2 U/ml (range of values 0.8—4.4). In concentrations of 10 to 40 U/ml SRS caused a potent bronchoconstriction lasting up to 30 min even with repeated washings with Tyrode solution. All of our preparations were sensitive to less than 10 U/ml of SRS and no signs of tachyphylaxis were observed. SRS in concentration of 20 U/ml caused bronchoconstriction approximately equivalent to that of 0.4 µg/ml acetylcholine (ACh) or histamine (H_1). In this concentration, in contrast to immediate ACh or H_1 action there was a latency period of about 1 min and 4—10 min were required before the peak of contraction was reached.

Atropine (1 µg/ml), mepyramine (1—10 µg/ml) and methysergide (5—25 µg/ml) had no effect on SRS action. Beta adrenergic receptor blockade with propranolol (1—25 µg/ml) had no effect on the SRS induced bronchoconstriction, nor did alpha adrenergic receptor blockade with phentolamine 5—25 µg ml les en it

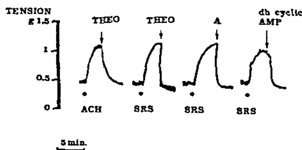


Fig. 1. Human bronchial strip suspended in a 4 ml bath. Effects of theophylline (THEO 50 µg/ml) adrenaline (A 1 µg/ml) and cyclic N^6 2' O-dibutyryl adenosine 3' 5'-monophosphate (db cyclic AMP 200 µg/ml) on slow reacting substance (SRS 25 U/ml) induced bronchoconstriction. Effect of the same dose of theophylline (50 µg/ml) on acetylcholine (ACh 0.4 µg/ml) induced constriction shown as comparison. After each washing of the bath 15 min were allowed for recovery.

TABLE I Antagonism by PPP of the SRS action on isolated human bronchi

PPP (μg/ml)	Dose ratio* (M ± SE)	Number of experiments
12 5	1 3 ± 0 2	10
50	2 2 ± 0 4	6
100	4 6 ± 0 8	6

* The dose ratio is the ratio of doses with and without antagonist to give equal responses (Gaddum *et al* 1955) Responses were measured 10 min after addition of the agonist

However adrenaline (0 1—1 μg/ml), theophylline (10—50 μg/ml) and cyclic N^6 2 O dibutyryl adenosine 3 5 monophosphate (db cyclic AMP, 50—200 μg/ml) counteracted the action of SRS The dilating effect of these substances is illustrated in Fig 1

PPP added to the bath 10 min before the agonist was tested in the concentration range of 12 5—100 μg/ml The effect of PPP on the SRS induced constriction is illustrated in Table I It shows the relationship (dose ratio) between the SRS dose required to produce the same constricting effect without PPP and in the presence of three concentrations of the antagonist in the bath The values used were obtained from dose response curves from 22 experiments In concentrations of 12 5 to 100 μg/ml PPP inhibited SRS induced bronchoconstriction but did not modify the effect of ACh and Hi on bronchi (Fig 2) Within this concentration range there was a parallel shift of the dose response curve

To complement the results obtained on bronchi some experiments were performed on the isolated guinea pig ileum which is the most commonly employed preparation to test the action of SRS Fig 3 shows the effect of three different PPP concentrations on the dose response curve of SRS The shift was parallel at 20 μg/ml but not at 40 and 80 μg/ml of PPP in the bath This finding indicates that PPP in high concentrations produces also a noncompetitive blockade of SRS action on guinea pig ileum

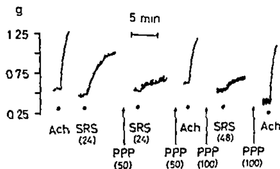


Fig 2 Human bronchial strip suspended in a 4 ml bath Inhibitory effect of polyphloretin phosphate /PPP (50) 50 μg/ml PPP (100) 100 μg/ml/ on bronchoconstriction induced by slow reacting substance (SRS (24) 24 U/ml SRS (48) 48 U/ml/ Constricting effect of acetylcholine (Ach 0 4 μg/ml) not modified by addition of PPP PPP added to the bath 10 min before the agonist

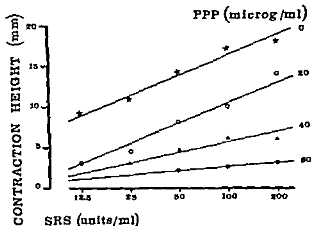


Fig 3 Isolated guinea pig ileum suspended in a 4 ml bath Dose response curves for the slow reacting substance (SRS) induced contractions without polyphloretin phosphate (PPP) and with PPP in concentrations 20, 40 and 80 μg/ml in the bath

Discussion

The SRS used in the present experiments is separated from prostaglandins (Änggård and Strandberg 1971) Cat paw SRS has similar biological and chemical properties as the SRS formed in anaphylactic reaction in guinea pig lung tissue (Chakravarty 1959, Anggård *et al* 1963) In previous studies of the bronchoconstricting action of SRS (SRS A guinea-pig lung) a more impure SRS, referred to as 'histamine free perfusate", has been used (Brocklehurst 1956, Berry and Collier 1964) Since $PGF_{2\alpha}$ is released in anaphylaxis in guinea pig lung (Piper and Vane 1969) and constricts the tracheobronchial tree it could not be unequivocally concluded that this SRS (SRS-A) possessed a bronchoconstricting effect. The present results demonstrate that a purified prostaglandin free cat paw SRS causes a marked constriction of isolated human bronchial muscle

The bronchoconstricting effect of SRS does not seem to be mediated via the adrenergic cholinergic histaminergic or 5-hydroxytryptaminergic receptors as explored by the use of antagonists SRS A has been claimed to release prostaglandins from the guinea pig lung (Piper and Vane 1969) However, since in our study no signs of tachyphylaxis in the response to SRS were observed its effect is unlikely to be secondary to the formation/release of prostaglandins

In addition to the earlier demonstrated antagonism of the $PGF_{2\alpha}$ action on isolated bronchi (Mathe Strandberg and Åstrom 1971) PPP also inhibits the effect of SRS However, four times higher concentration of PPP was needed to produce an equivalent inhibition of the bronchoconstricting effect of SRS Since antagonism by PPP could be surmounted by higher doses of agonists and there was a parallel shift of the log dose response curves of the agonists, it seems that PPP blocks SRS and $PGF_{2\alpha}$ on a competitive basis PPP has been shown to inhibit a variety of enzymes, *e g* alkaline phosphatase hyaluronidase and urease (Diczfalusy *et al* 1953) However it does not seem likely that inhibition of bronchoconstriction induced by

and SRS is due to this mechanism of action (Eakins 1970) Thus some phosphorylated polymers having similar enzyme inhibitory properties had no prostaglandin blocking activity It should be mentioned that certain N phenylanthranilic acids, *e g* meclofenamate antagonize the constricting effect of SRS A and $PGF_{2\alpha}$ on isolated human bronchi (Collier and Sweatman 1968) SRS and $PGF_{2\alpha}$ are unsaturated biologically active hydroxy acids The fact that the action of both substances is antagonized by PPP would seem to further indicate their structural similarity In this context it is interesting to note that PPP is also an acidic compound with hydroxylic groups

Thus PPP has been shown to antagonize bronchoconstricting effects of SRS and $PGF_{2\alpha}$ two substances that may play a role in bronchial asthma (Brocklehurst 1962 Austen 1965 Piper and Vane 1969) Since PPP has relatively low toxicity (Hogberg *et al* 1953, Fries 1956) the present finding merits further investigation of PPP's mechanism of action

We wish to express our thanks to Professor V O Bjorck and to Dr D I F Rodriguez from the Department of Thoracic Surgery Karolinska sjukhuset Stockholm Sweden for supplying the lungs and to Dr B Hogberg AB Leo Halsingborg Sweden for supplying PPP This investigation was supported by a grant from the Leo Research Foundation which is gratefully acknowledged

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From the Institute of Medical Physiology, University of Copenhagen Denmark

Stability of [Mg^{2+}] in Cerebrospinal Fluid during Plasma Changes and during Hypercapnia in Young and in Adult Rats

By

O M Olsen and S C Sorensen

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Abstract

Olsen O M and S C Sorensen *Stability of [Mg^{2+}] in cerebrospinal fluid during plasma changes and during hypercapnia in young and in adult rats* Acta physiol scand 1971 *82* 466—469

This investigation was undertaken to examine whether the rat like other mammals that have been studied maintains a stable concentration of Mg^{2+} in cerebrospinal fluid (CSF) when the plasma Mg^{2+} concentration is changed CSF was sampled from the cisterna magna in adult [illegible] The [Mg^{2+}] in CSF did not [illegible] previous findings in the rat [illegible]ge of the animals when they [illegible]tely developed in young rats [illegible]ut we found that rats 3 to 4 weeks old also maintained a stable [Mg^{2+}] in CSF during hypo[illegible]agnesemia The effect of 4 hrs of hypercapnia on the [Mg^{2+}] in CSF was examined in normal [illegible] hypomagnesemic adult rats Hypercapnia did not affect the [Mg^{2+}] in CSF in either group

The concentration of Mg^{2+} in cerebrospinal fluid (CSF) of mammals is maintained remarkably constant when the plasma concentration of Mg^{2+} is varied (McCance and Watchorn 1932 Kemeny Boldizsar and Pethes 1961 Oppelt MacIntyre and Rall 1963 Bradbury *et al* 1968) However Chutkow and Meyers (1968) reported a pronounced decrease in [Mg^{2+}] in CSF when rats were maintained hypomagnesemic for several days This finding was inexplicable to us because of the stability of [Mg^{2+}] in CSF of other mammalian species which have been studied and we therefore re-examined whether the [Mg^{2+}] in CSF changes in the rat during variations in the plasma concentration of Mg

When we failed to observe a fall in [Mg^{2+}] in CSF during hypomagnesemia we wondered whether the difference between our findings and those of Chutkow and Meyers (1968) who studied young rats could be due to incomplete development of the blood brain barrier in young rats (Vernadakis and Woodbury 1965) We therefore repeated the study in rats 24 to 27 days old

The stability of [Mg^{2+}] in CSF during variations in the plasma concentration of Mg^{2+} indicates that the rate of active transport of Mg^{2+} across the blood brain barrier

changes to compensate for variations in the passive flux of the ion (Oppelt *et al* 1963, Bradbury 1971) Augmentation of the permeability of the blood brain barrier to the ions will increase the passive influx of Mg^{2} from blood to brain extracellular fluid Because the permeability of the blood brain barrier increases during hypercapnia (Clemedson Hartelius and Holmberg 1958, Lending Slobody and Mestern 1961, Cutler and Barlow 1966, Cameron, Davson and Segal 1970) we used hypercapnia as a way to investigate whether the system can maintain a stable $[Mg^{2}]$ in CSF under induced increases in the permeability of the blood brain barrier

Methods

Intyre and Davidsson water (2 1/2 g/liter) Plasma was separated visible hemolysis were discarded CSF was sampled from cisterna magna It was possible to withdraw 75—125 μl CSF in about 90 % of the rats Samples which were not clear were discarded

The magnesium analysis was performed on an Atomic absorption spectrophotometer (Perkin Elmer Model 290) Recovery averaged 100 8 %, coefficient of variation = 2 9 % n 10 Plasma pH was measured in a pH electrode (Radiometer Copenhagen)

Hypercapnia was induced by placing four rats in a box through which 15 % CO in air flowed at 800 ml/min After 4 hrs the animals were removed from the box and anesthetized with pentobarbital (20 mg/kg) for sampling of CSF and blood The trachea was immediately cannulated and the animals were ventilated with the same gas mixture that they breathed in the box To avoid the increased bleeding into CSF during sampling when the P_{CO_2} was high we ventilated the animals with room air for a few minutes before the CSF sampled the animals were again ventilated with the hypercapnic mixture for at least 3—5 min before a blood sample was obtained

Results

$[Mg^{2}]$ in CSF Two groups of rats were studied One group was 10 to 12 weeks old and weighed about 200 g when they entered the experiment The other group were 24 to 27 days old and weighed 50—70 g In the older animals CSF samples were obtained 11 and 43 days after they were put on a magnesium free diet In the young animals CSF samples were obtained after 8 and 14 days of dieting Because there was no difference in the magnesium concentration in CSF between the early and the late samples in either age group the data were pooled Fig 1 shows the rela

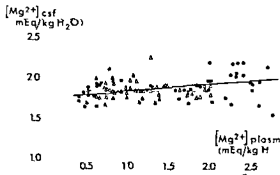


Fig 1 The relationship between $[Mg^{2}]$ in plasma and CSF in young (●——) and adult ▲) rats The lines are the linear regression lines for all points in each group

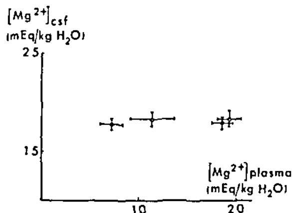


Fig. 2. The effect of 4 hrs of hypercapnia (open sympols) on $[Mg^{2+}]$ in plasma and CSF in normal rats (n = [illegible]

hrs in the box. The horizontal and vertical bars depict ±1 standard deviation.

tionship between $[Mg^{2+}]$ in CSF and plasma in the two groups of animals. In both groups the CSF concentration remains very stable despite large variations in the plasma concentration. The difference between the slopes of the regression lines might reflect a true difference between the two groups, but the difference is highly insignificant ($p<0.001$) by analysis of covariance.

The effect of hypercapnia on $[Mg^{2+}]$ in CSF. The effect of hypercapnia on $[Mg^{2+}]$ was studied both in normal rats and in rats who were fed a magnesium-free diet for 45 days. These rats all weighed more than 200 g. In neither group did hypercapnia have any significant effect on the $[Mg^{2+}]$ in CSF as illustrated in Fig. 2. In the hypomagnesemic rats there was a significant increase in $[Mg^{2+}]$ in plasma during the four hours of hypercapnia. During hypercapnia pH in plasma fell from 7.36 ± 0.05 (SD) to 7.04 ± 0.02 (SD) in the normal rats and from 7.44 ± 0.03 (SD) 7.10 ± 0.02 (SD) in the hypomagnesemic rats.

Discussion

We reexamined if the rat maintain a stable concentration of Mg^{2+} in CSF when the plasma concentration varies, because Chutkow and Meyers (1968) found that rats did not do so during prolonged hypomagnesemia. In contrast, man, rabbits and dogs maintain a stable $[Mg^{2+}]$ (McCance and Watchorn 1932, Kemeny *et al.* 1961, Oppelt *et al.* 1963, Bradbury *et al.* 1968). We found that the rat is similar to other mammals in this respect so we then examined if the difference between our findings and those reported by Chutkow and Meyers (1968) could be explained by a difference in age of the animals, but our young animals, which were presumably younger than those used by Chutkow and Meyers (1968), also maintained a stable $[Mg^{2+}]$ in CSF during chronic changes in $[Mg^{2+}]$ in plasma. The finding is compatible with the view that the cation transport processes of the blood-brain barrier is developed at birth as suggested by Bito and Myers (1970) who studied the development of adult CSF/plasma ratios for Mg^{2+}, Ca^{2+} and K^+ in the rhesus monkey.

Hypercapnia experiments The constancy of $[Mg^{2+}]$ in CSF of rats exposed to 15 % CO_2 for 4 hrs indicate either that the permeability of the blood brain to this ion did not change or that the increase in passive flux of Mg^{2+} did not exceed the capacity of the transport system responsible for maintaining a stable $[Mg^{2+}]$ in CSF Even in hypomagnesemic animals there were no changes in CSF during hyper capnia although the presumed increase in permeability was superimposed upon a larger concentration difference between blood and CSF

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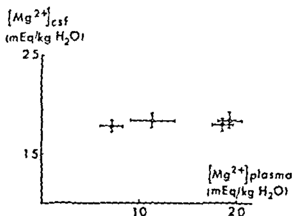


Fig 2 The effect of 4 hrs of hypercapnia (open symbols) on $[Mg^{2+}]$ in plasma and CSF in normal rats (n = ... hrs in the box The horizontal and vertical bars depict ±1 standard deviation

tionship between $[Mg^{2+}]$ in CSF and plasma in the two groups of animals In both groups the CSF concentration remains very stable despite large variations in the plasma concentration The difference between the slopes of the regression lines might reflect a true difference between the two groups but the difference is highly insignificant ($p < 0.001$) by analysis of covariance

The effect of hypercapnia on $[Mg^{2+}]$ in CSF The effect of hypercapnia on $[Mg^{2+}]$ was studied both in normal rats and in rats who were fed a magnesium free diet for 45 days These rats all weighed more than 200 g In neither group did hypercapnia have any significant effect on the $[Mg^{2+}]$ in CSF as illustrated in Fig 2 In the hypomagnesemic rats there was a significant increase in $[Mg^{2+}]$ in plasma during the four hours of hypercapnia During hypercapnia pH in plasma fell from 7.36 ± 0.03 (SD) to 7.04 ± 0.02 (SD) in the normal rats and from 7.44 ± 0.03 (SD) 7.10 ± 0.02 (SD) in the hypomagnesemic rats

Discussion

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TABLE II Body weights and total water contents of obese-hyperglycemic mice and lean litter mates at different ages The figures indicate mean values ± S E M The figures within brackets denote the number of animals

Age	Obese animals		Lean animals	
	Body weight (g)	Water content (% of wet weight)	Body weight (g)	Water content (% of wet weight)
18 d (5)	12.4 ± 1.5	64.0 ± 3.5	10.2 ± 0.8	71.4 ± 0.5
1 m (6)	19.6 ± 1.1	53.6 ± 1.0	16.7 ± 0.7	71.2 ± 0.4
3—4 m (6)	46.0 ± 1.3	40.6 ± 1.0	26.9 ± 1.1	62.7 ± 2.0
7 m (7)	58.5 ± 2.1	38.5 ± 1.5	37.9 ± 1.4	60.7 ± 1.2

B The body weights and water content of the bodies of obese and lean mice at different ages are shown in Table II In the youngest age group 18 days old animals, the homozygous mice were identified by their enlarged intraabdominal fat depots The mean body weight of the homozygous animals still had not reached levels significantly above that of the lean controls The amount of body water did not differ significantly between the two groups At an age of 1 month, when the homozygous mice displayed a probably significant increase of body weight as compared with the lean litter mates ($P < 0.05$), *the percentage water content of the body* was reduced The difference between the two types of mice was even more pronounced at 3—4 months of age In 7 months-old mice the difference in body weight between the obese and lean animals was 20.6 However, the total amount of water at the age of 7 months was about the same in obese mice as in their lean litter mates

C The results of the study on the sulfate space are shown in Fig 1 Mixing of the injected ^{35}S sulfate with the rapidly miscible sulfate pool appeared almost complete after approximately 30—45 min The rate of fall in plasma concentration of ^{35}S-sulfate was about the same in obese and lean mice

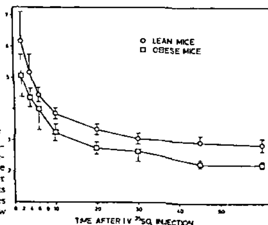


Fig 1 *Radioactivity of plasma at different* times after *i v* injection of ^{35}S-sulfate into nephrectomized obese-hyperglycemic and lean mice The ordinate indicates DPM $^{35}S \times 10^{-4}$ per 10 µl plasma Each symbol represents the mean value for the plasma samples from 5 animals Vertical bars show S E M

TABLE III Body weight and sulfate of adult lean and obese hyperglycemic mice Figures are mean values ± S E M

	number of mice	body weight g	sulfate space ml	sulfate space in % of body weight
lean mice	5	30 8±0 8	8 8±0 6	28 4±1 2
obese mice	5	53 3±1 3	11 2±0 5	21 0±0 8

The sulfate space of each animal was calculated from the ^{35}S sulfate concentration of plasma samples at 60 min and the total amount of ^{35}S injected Table III shows the sulfate spaces in ml and in per cent of the body weights The total sulfate space expressed in ml was significantly larger ($p < 0\ 005$) in obese mice than in lean controls the difference being approximately 27 % or on the average about 2 4 ml The corresponding difference in body weight between the two groups was about 73 per cent The sulfate space per unit body weight was however considerably lower in the obese mice ($P < 0\ 0005$) About 0 87 ml of the increased sulfate space in the obese animals was accounted for by the larger blood volume — the space due to penetration of sulfate into the erythrocytes is included in this figure The remaining additional sulfate space is about 1 5 ml which corresponds to an extravascular sulfate space of 64 µl/g extra fat tissue in the obese mice if other tissues are assumed to be equal in obese and lean animals

D The *in vitro* study of the penetration of sulfate into erythrocytes showed rapid penetration of $^{35}SO_4$ into the cells The erythrocyte plasma ratio of radioactive sulfate was 0 31 and 0 32 in obese and lean mice respectively after an incubation time of 20 min After 60 min the corresponding values were 0 33 and 0 30 respectively In the *in vivo* experiments 10 min after the injection of $^{35}SO_4$ the erythrocyte plasma ratio in pooled blood from 3 animals was 0 34 At 60 min it was 0 33 in pooled blood from 3 animals All determinations of ^{35}S were made in triplicate The results agreed within 5 %

Discussion

From the present data it is apparent that in mice with the hereditary obese hyperglycemic syndrome the increased body weight is associated with an enlarged blood volume The observed increase in blood volume in the *obob* mice is larger than that reported by Yen Steinmetz and Simpson (1970) The animals used by these investigators were 3 1/2 months old while our animals were 5 months of age The difference in age may explain the discrepancy The mean value for the blood volume per unit body weight in obese animals was reduced both in our experiments and those of Yen *et al* (1970) These observations suggest that the amount of blood is somewhat less in adipose tissue than in so-called lean body mass

The size of sulfate space in the lean animals approximately 28 % of the body weight is in good agreement with values found in other species *i e* rats dogs and humans (*cf* Dittmer 1961) Sulfate penetrates into erythrocytes in mice as shown

in the present study and therefore does not give an exact estimate of the extracellular space

The sulfate space was enlarged in the obese mice In human adults the extracellular fluid in newly deposited obesity tissue has been found to be 13—15 % per unit weight (Keys Andersson and Brozek 1955) The extracellular space of the obesity tissue of the *obob* mice in our study can be calculated to about 10 %

The value for the body hematocrit being 82 % of the orbital vein hematocrit in both obese and lean mice is lower than the 88 % reported for man (Larsen 1968) rats (Everett Simmons and Lasher 1956) and mice (Wish Furth and Storey 1954) As pointed out under Results the ratio body hematocrit large vein hematocrit in our study may have a small error due to the fact that the orbital vein hematocrit was determined at the end of the experiments (At that time relatively large volumes of blood could be obtained without adverse effects on the study on the loss of labelled material from the general circulation) There probably are several reasons for the difference between body and large vein hematocrits axial streaming of red blood cells in small vessels rapidly equilibrating extravascular albumin spaces and plasma skimming into narrow capillaries (Palmer 1965 Everett Simmons and Lasher 1956 Larsen 1968) The similarity in ratio body hematocrit large vein hematocrit between obese and lean animals indicates that the fat tissue responsible for the increase in body weight had a hematocrit that was about the same as that in the lean body mass

In both obese and lean mice the mixing of the labelled red blood cells in the circulating blood appeared to be almost complete within 3 min Nor were there any marked differences in the rates of albumin and sulfate loss from the vascular to the extravascular pools in the two types of animals These results indicate that the accumulated adipose tissue is not a region with sluggish blood flow and/or low rate of albumin and sulfate turnover in the extravascular pools

Sulfate permeability of the erythrocyte membrane varies between species Sulfate penetrates into erythrocytes from cattle dog and goat (Denis and Reed 1926 Eichler 1950) but not into human and sheep red blood cells Reed and Denis 1927, Woodhouse and Pickworth 1932 Bourdillon and Lavietes 1936) The present *in vivo* and *in vitro* experiments yielded erythrocyte plasma ratio for sulfate of 0 3 which is in agreement with the results obtained in cattle dog and goat

A recent study has shown that the amount of inorganic miscible sulfate in obese hyperglycemic mice is proportional to the body weights of the animals (Herbai 1970 b) In obese animals with a body weight of 53 3 g the amount of sulfate expected from the regression line is about 750 μg In lean controls with a body weight of 30 8 g the expected amount of sulfate is 400 μg The results reported here indicate that the sulfate space in obese animals is only 27 % larger than that in lean controls Taken together the results suggest that the sulfate concentration in plasma and the interstitial tissue fluid is larger in obese animals than in lean mice Direct determinations of the concentrations of sulfate in plasma are necessary however to establish whether hypersulfatemia is a characteristic for obese hyperglycemic mice

The results of the present study show that in comparisons of drug effects in adult obese mice and their lean litter mates one has to consider the distribution space of the drug in question and that this is the case not only for lipid soluble agents A water-soluble agent which is strongly bound to plasma protein or which in itself has a very high molecular weight will attain comparable plasma concentrations if obese animals receive about 50 per cent more of the agent than lean mice A drug with even distribution within the whole extracellular space will attain about the same concentration within its space if the dose in obese animals is 25 % larger than that given to lean mice Finally, an agent that becomes distributed throughout the whole body water will reach about the same concentration within its space if equal amounts of the agent are given to obese and lean animals

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From the Department of Anatomy, Karolinska Institutet, Stockholm, Sweden

Postnatal Excitability Changes of the Ankle Monosynaptic Reflexes in the Cat

By

ANDERS MELLSTRÖM

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Abstract

MELLSTRÖM, A *Postnatal excitability changes of the ankle monosynaptic reflexes in the cat* Acta physiol scand 1971 82 477—489

The effects of repetitive stimulation on the amplitude of ankle extensor and flexor monosynaptic reflexes were studied under various experimental conditions in high spinal cats during postnatal development. The extensor monosynaptic reflex elicited from a dorsal root at 1/9 sec decreases initially when the stimulus rate is increased to 2/sec This depression increases with age and is more pronounced in the flexor monosynaptic pathway than in the extensor one in the newborn kitten This latter observation and the demonstration of a subliminal fringe in the flexor motoneurone pool of the newborn kitten indicate an earlier maturation of the flexor monosynaptic pathway In young kittens the extensor monosynaptic reflex decreases less with increasing rate of stimulation when elicited from a dorsal root than from a peripheral nerve In older kittens the reverse is true Prolonged stimulation reduces the differences between dorsal root and peripheral nerve stimulation In young kittens prolonged dorsal root stimulation at 2/sec causes a decrease of the extensor monosynaptic response In adult animals on the other hand it increases The flexors show a passing increase and subsequent decrease in young kittens during prolonged stimulation and in the adult there is only a decrease Nembutal® depresses the extensor monosynaptic potential and accentuates frequency-dependent depression variably with age

In light of results recently published about the postnatal growth changes of lumbar motoneurones (Mellstrom and Skoglund 1969) and their postnatally changing synaptology (Conradi and Skoglund 1969) it appeared of interest to study the reflex transmission in spinal monosynaptic pathways during postnatal development under various experimental conditions

The monosynaptic potential in extensor muscle nerves shows no early facilitation after supramaximal homosynaptic conditioning in the newborn Nembutal® anesthetized kitten The period of complete unresponsiveness lasts from 10 to 110 msec (Skoglund 1960 b) as against 10 to 30 msec preceded by facilitation in the adult cat (Brooks Downman and Eccles 1950) In the newborn kitten the total period of recovery was found to be at least 7 sec (Skoglund 1960 b) In the adult cat this period is longer than 660 msec (Brooks *et al* 1950) but does not exceed 3 sec (Eccles and

Rall 1951, Jefferson and Schlapp 1953) Lloyd and Wilson (1957), however, claimed that the depression lasted for as long as 20 sec

Wilson (1962) mainly confirmed Skoglund's finding (1960 b) about the recovery cycle in the monosynaptic pathway of the newborn and showed that low frequency depression (see Brooks *et al* 1950, Lloyd and Wilson 1957) is the same whether elicited by peripheral nerve or dorsal root stimulation Wilson (1962) also stated that low-frequency depression is 'somewhat deeper in the younger animals"

In this study the postnatal changes of low frequency depression have been compared in ankle extensor and flexor monosynaptic pathways The monosynaptic reflexes were elicited by dorsal root stimulation The extensor monosynaptic reflex was also compared when set up from a dorsal root and a peripheral nerve A prerequisite for this study is an unanesthetized animal and therefore high spinalized kittens and cats were used The effects of anesthesia were, however, also studied to allow comparisons with earlier results It will be shown that the monosynaptic reflex *is initially more depressed when the stimulation frequency is increased and that this* effect increases with age and under the influence of Nembutal® The postnatal development of extensor and flexor monosynaptic pathways is shown to be different

Material and Methods

Results from some 40 kittens ranging in age from newborn to two and a half months and from 5 adults cats are reported The animals were anesthetized with ether and a tracheal cannula was inserted The spinal cord was sectioned between the first and second cervical vertebrae and the animals were artificially ventilated The ventilation frequency was adapted to the spontaneous respiration frequency of the kittens which decreases with increasing age (e g Schwieler 1968

The animals were immobilized with Flaxedil® i p (Eccles Eccles and Fatt 1956 Wilson 1962) A lumbar laminectomy was performed and the dorsal roots L4 S4 on one side were severed The medial and lateral gastrocnemius nerves the plantaris nerve (ankle extensor) the anterior tibial nerve and the short and long extensor digitorum nerves (ankle flexor) were dissected free in the popliteal fossa on the same side as the severed roots

The dorsal root L7 or S1 was stimulated supramaximally with square waves of 0 3—0 5 msec duration through an isolation transformer and the monosynaptic potential recorded from the prepared peripheral muscle nerves

To assure a stable monosynaptic response in the younger kittens recording did not start until at least 2 5 hrs had elapsed following spinalization and discontinuation of ether anesthesia In the older animals 4—5 hrs for the same reason usually had to elapse before recording started. The rectal temperature of the animals was kept between 37 5 and 38 5° C by external heating

In some spinalized kittens of varying ages the ventral roots L4 to S4 on the opposite side as well as the corresponding dorsal roots except L7 and S1 were also cut The same peripheral extensor muscle nerves as those mentioned above were dissected free on this side too The monosynaptic potential was here elicited by supramaximal stimulation of the peripheral nerves and recorded on the ventral roots L7 or S1 It was thus possible to compare the effects on the amplitude of the monosynaptic potential caused by both dorsal root and peripheral nerve stimulation in one and the same animal However according to Wilson 1962 (see also Eccles Shealy and Willis 1963 heterosynaptic reflexes are elicited by single stimuli in the newborn and young animal when stimulating peripheral nerves and recording from ventral roots and thus the monosynaptic potentials from roots and peripheral nerves are not strictly comparable In order to study the effects of Nembutal® it was sometimes given i p in dosages of 35—45 mg/kg b w after the comparison of dorsal root and peripheral nerve stimulation had been completed This comparison was then repeated some 30 min after the administration of the anesthetic agent.

The monosynaptic potential was photographed on bromide film from an oscilloscope beam The amplitude of the monosynaptic potential was measured from the base line to the peak of

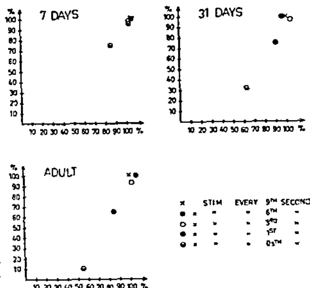


Fig 1 Relative changes of amplitude (ordinate) and width (abscissa) of the monosynaptic potential at different stimulation rates In this and Figures 2, 3 and 5—8 each point is the mean of the 11 initial potentials except the first one at each rate The mean values at stimuli 1/9 sec equals 100 % Stimulation of the dorsal root L7 and recording from the plantaris nerve

the spike In order to exclude the possibility that changes of the amplitude were negatively correlated with changes in shape of the base of the potential, the height and the width of the potentials were compared in some experiments Fig 1 shows that the width at the base and the amplitude are never negatively correlated

The effect on the monosynaptic potential of the following stimulation frequencies was systematically investigated 1/9 sec 1/6 sec, 1/3 sec 1/sec and 2/sec Sometimes frequencies higher than 2/sec were used An increased variation in amplitude from potential to potential with … monosynaptic poten… recorded at a stimu… *initial* depression or … stimulation frequency were used The first one was omitted since it usually has a larger amplitude (*cf* Fuortes and Hubel 1956 Lloyd and Wilson 1957) When the effect of a more *prolonged* stimulation is reported it refers to some 60 sec stimulation Changes less than ± 10 % from the control have generally not been regarded as significant (*cf* Jefferson and Schlapp 1953 Hunt 1955 Somjen and Heath 1966 Rudomin and Dutton 1969 a b)

Results

Changes of the extensor monosynaptic reflex in response to iterative stimulation

In the animal 6—8 hrs old no initial depression was seen (Fig 2) At somewhat older stages the depression is only evident at higher stimulation rates More than 10 % depression is as a rule not obtained until about 20 days postnatally at a stimulation frequency of 1/sec In animals more than 30 days old such a depression appears even with a stimulation frequency of 1/3 sec

From birth onward (Fig 3) there is a clear relative diminution of the amplitude of the potential at a stimulation frequency of 2/sec This initial depression seems to increase until the animal has reached an age of about 40 to 50 days At this time the relative decrease is 60—70 %. In older animals no further depression of the

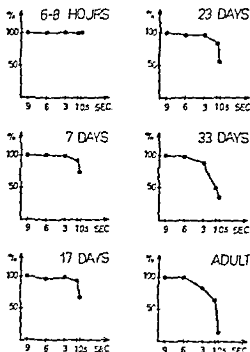


Fig 2 Relative changes of the amplitude of the ankle extensor monosynaptic potential (ordinate) versus interval in sec between iterative stimuli (abscissa) Stimulation of the dorsal root L7 and recording from the plantaris nerve

monosynaptic potential seems to occur Results comparable to those shown in Fig 3 were also obtained when recording from the medial and lateral gastrocnemius nerves When higher stimulation frequencies than 2/sec were used the depression was even more pronounced

From Fig 2 and Fig 3 is thus seen that the initial depression of the monosynaptic potential from extensors increases with increasing stimulation frequency and increasing age

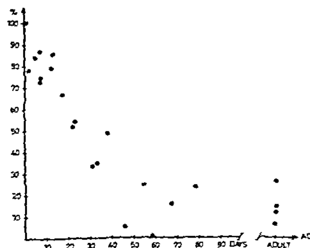


Fig 3 Relative changes of the amplitude of the ankle extensor monosynaptic potential at iterative stimuli 2/sec (ordinate) versus age abscissa Stimulation of the dorsal root L7 and recording from the plantaris nerve

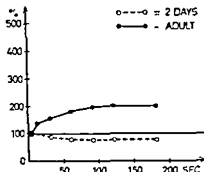


Fig 4 Relative changes of the amplitude of the ankle extensor monosynaptic potential during iterative stimuli 2/sec (ordinate) versus time in sec after stimulation onset (abscissa) Each point is the mean from ten potentials The mean from the eleven first potentials except the first one equals 100 % Stimulation of the dorsal root L7 and recording from the plantaris nerve

The described changes of the monosynaptic potential are as stated above the mean of the eleven first potentials except the first one at each stimulation frequency In order to see if those initial changes were stable the amplitude changes of the monosynaptic potential during a prolonged iterative stimulation were also studied The amplitude of the monosynaptic reflex in the adult cat is found to increase during prolonged stimulation, while in the newborn animal it decreases slightly (Fig 4) From these and other experiments it became evident that a steady state was reached after about one minute Compared with the amplitude at a stimulation frequency of 1/9 sec the steady state value was reduced by 15—20 % in kittens older than 2 months Earlier, it was reduced by 30—50 % except in the newborn animal where no reduction at all was seen

Comparison of extensor monosynaptic reflexes elicited from a dorsal root and a peripheral nerve The effects of anesthesia

In some cases the monosynaptic potential elicited from the dorsal roots and recorded on a peripheral nerve on one side was compared with the potential elicited from the corresponding peripheral nerve on the other side and recorded from a ventral root Comparisons were made at varying stimulation frequencies both before and after Nembutal® anesthesia The amplitude of the monosynaptic potential in the 12-day-old kitten (Fig 5 A) was more depressed initially at the highest stimulation frequency used, when elicited from an ankle extensor nerve and recorded on a ventral root than if elicited from a dorsal root and recorded on an ankle extensor muscle nerve Under the same conditions in the 75-day-old kitten (Fig 5 C) however the initial depression of the monosynaptic potential was larger when elicited from the dorsal root than from the peripheral nerve It might thus be concluded that the monosynaptic reflex elicited from a peripheral nerve is more depressed than when elicited from a dorsal root with increasing stimulation frequency in the early stages of development With increasing age the reverse becomes true

Some 30 min after giving Nembutal® 35—45 mg/kg b w i p the above mentioned comparisons were repeated (see right side, Fig 5) The absolute amplitude of the monosynaptic potential was found to decrease under the influence of anesthesia (*cf*

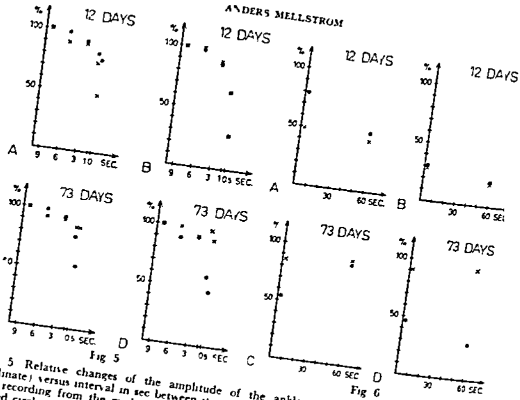


Fig 5

Fig 6

Fig 5 Relative changes of the amplitude of the ankle extensor monosynaptic potential (ordinate) versus interval in sec between iterative stimuli (abscissa) Stimulating a dorsal root and recording from the medial and lateral gastrocnemius nerves on one side of the animal (filled circles and on the other side stimulating the medial and lateral gastrocnemius nerves and recording from a ventral root (crosses) A and C before B and D after giving 35—45 mg/kg b w i p of Nembutal®

Fig 6 Relative changes of the amplitude of the ankle extensor monosynaptic potential during iterative stimuli 2/sec (ordinate) versus time in sec after stimulation onset (abscissa) Stimulating a dorsal root and recording from the medial and lateral gastrocnemius nerves on one side of the animal (filled circles) and on the other side stimulating the medial and lateral gastrocnemius nerves and recording from a ventral root (crosses) A and C before B and D after giving 35—45 mg/kg b w i p of Nembutal®

Weakly 1969) and the monosynaptic potential in the 12 day-old kitten (Fig 5 B) was relatively more depressed initially with increasing stimulation frequency This was especially true with dorsal root stimulation On the other hand there was no longer any difference between dorsal root and peripheral nerve stimulation (cf Eccles and Willis 1965) Dorsal root stimulation in the anesthetized 73 day-old kitten revealed a slightly greater initial depression of the monosynaptic potential as compared to the unanesthetized animal (Fig 5 D) The potential elicited from the peripheral nerve on the other hand showed no further depression from its reduced size after Nembutal® as compared with the reduction seen with iterative stimulation in the unanesthetized state From these findings can be concluded that Nembutal® decreases the absolute amplitude of the monosynaptic potential as compared with that in the unanesthetized animal irrespective of age tested Under anesthesia the difference in relative reaction to iterative stimulation between the monosynaptic

potentials elicited from the dorsal root and peripheral nerve disappeared in the young animals, whereas in the older animals it remained mainly unchanged

When Nembutal® 35—45 mg/kg b w was given i p a total depression of the monosynaptic potential occurred at a lower frequency of iterative stimulation than without anesthesia in the younger kittens In a 12-day-old unanesthetized kitten a total depression of the monosynaptic potential was obtained when stimulating the peripheral nerve with a frequency of 20/sec About half an hour after Nembutal® had been given this was obtained with a frequency of 10/sec (*cf* Skoglund 1960 b) The same was true when stimulating o dorsal root A total depression of the monosynaptic potential elicited from the dorsal root in the same animal could, however, not be obtained before Nembutal® was given, even with a stimulation frequency of 100/sec It is thus obvious that a total depression of the monosynaptic potential is obtained at a lower frequency when it is elicited from a pheripheral nerve than from a dorsal root in the unanesthetized preparation The results of peripheral nerve stimulation in the anesthetized preparations are in good agreement with those of Skoglund (1960 b), although his observatins were made only in newborn animals

In a 12 day-old unanesthetized kitten (Fig 6 A) the mean amplitude decreased less than 10 % when stimulating the peripheral nerve 2/sec, during 60 sec but by about 30 % when stimulating the dorsal root In an unanesthetized 73 day-old cat (Fig 6 C) no changes were obtained with prolonged peripheral nerve stimulation at a stimulation rate of 2/sec while dorsal root stimulation during 60 sec gave an increase by 30 % of the initially recorded amplitude This indicates that, compared with the initially recorded amplitudes a prolonged stimulation of a peripheral muscle nerve gives a small further depression of the monosynaptic potential in the young stages and no change at all in the older animal Prolonged stimulation of a dorsal root gives a further depression of the monosynaptic potential in the young animal but an enhancement in the older animal

When the above comparison is repeated after administration of Nembutal® (right side Fig 6) the changes of thus reduced potentials in response to prolonged stimulation gave a further decrease of some 10 % following both dorsal and peripheral nerve iterative stimulation in the 12 day old animal (Fig 6 B) In the 73 day old animal (Fig 6 D) prolonged stimulation of the peripheral nerve increased the monosynaptic potential less than 5 % whereas dorsal root stimulation gave a further depression of 15 %

Changes of the flexor monosynaptic reflex in response to iterative stimulation

The studies of the monosynaptic reflex have been somewhat hampered by the difficulty of obtaining stable flexor monosynaptic potentials in spinal animals older than three to four postnatal weeks The experimental data with regard to the flexors in older animals are therefore somewhat limited and no comparisons have been made between dorsal root and peripheral nerve stimulation

From Fig 7 it is apparent that during the first three to four postnatal weeks there is hardly any change of the initial depression with increasing stimulatio

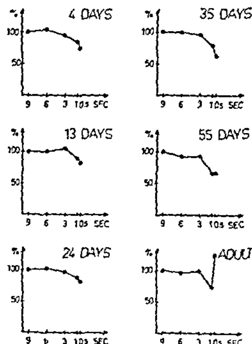


Fig 7 Relative changes of the amplitude of the ankle flexor monosynaptic potential (ordinate) versus interval in sec between iterative stimuli (abscissa) Stimulation of the dorsal root L7 and recording from the long and short extensor digitorum nerves

frequency Between the first and second month there is a small increase of the depression At the end of the second month the difference in the degree of depression between the two highest stimulation frequencies is, if any, very small In the adult animal there is a clear initial increase of the amplitude when the stimulation rate is changed from 1/sec to 2/sec

The initial depression of the flexor monosynaptic potential at a stimulation frequency of 2/sec shows a great variation during the first two postnatal months (Fig 8) In the adult cat on the other hand a relative initial increase is obtained (cf Bernhard 1945 Fuortes and Hubel 1956) However this increase (Fig 7 and 8) declines when the stimulation is continued (Fig 9) An initial increase of the monosynaptic reflex at a stimulation frequency of 2/sec is not obtained in animals less than 2 months of age (Fig 7) In the newborn animal the amplitude of the monosynaptic potential also increases if the stimulation is continued to reach a peak around 30 sec later and then slowly declines (Fig 9 If the peak value is compared with the amplitude when stimulating 1/9 sec it is found to exceed the latter by some 35 % This indicates the presence of a subliminal fringe in the flexor motoneurone pool in the newborn stage, since no heterosynaptic reflexes are included when stimulating a dorsal root and recording from a peripheral nerve (cf Beswick and Evenson 1955) If the monosynaptic potential in the steady state of prolonged iterative stimulation at a frequency of 2/sec is compared with the one at a frequency of 1/9 sec it is generally found to be somewhat depressed but less in the adult than in the young animals

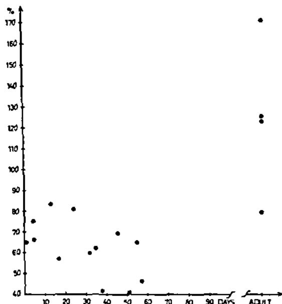


Fig 8 Relative changes of the amplitude of the ankle flexor monosynaptic potential at iterative stimuli 2/sec (ordinate) versus age (abscissa) Stimulation of the dorsal root L7 and recording from the long and short extensor digitorum nerves

From these experiments it can thus be concluded that stable monosynaptic potentials from flexors are as a rule more easily obtained during the first postnatal month than later on Furthermore, the flexor monosynaptic potential recorded from a peripheral nerve in response to iterative dorsal root stimulation is initially more depressed at birth than the extensor monosynaptic potentials when faster stimulation frequencies are used The initial depression of the flexor also increases less than that of the extensor with increasing age

As shown in Fig 4 and 9 there is also a difference between extensor and flexor monosynaptic potetials with regard to prolonged iterative stimulation In the young animal the extensor response decreases This is also the case with the flexor after showing a passing increase during the first minute although its steady state value is less depressed than that of the extensor In the adult the extensor response increases but the flexor decreases

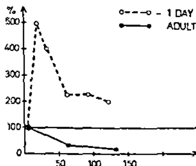


Fig 9 Relative changes of the amplitude of the ankle flexor monosynaptic potential during iterative stimuli 2/sec (ordinate) versus time in sec after stimulation onset (abscissa) Each point is the mean from ten potentials The mean from the eleven first potentials except the first one equals 100 % Stimulation of the dorsal root L7 and recording from the long and short extensor digitorum nerves

Discussion

The increasing initial depression with increasing stimulus rate and age will be discussed in the light of the results obtained when comparing peripheral nerve and dorsal root stimulation as well as those obtained when using Nembutal® It is possible that the use of spinalized unanesthetized animals has helped to disclose the differences in maturity between flexors and extensors The study of the effects of anesthesia however are helpful in explaining some of the results obtained In both cases postnatal changes in the supraspinal influence on the spinal reflex patterns were under all circumstances excluded (*cf* Skoglund 1960 a Ekholm 1967) On the other hand Flaxedil® had to be used but no effects of this drug on the mechanisms under study were observed Thus when the concentration of Flaxedil® decreased until muscular movements appeared no changes in the recorded responses were encountered The deep rectal temperature of the animal was maintained between 37 5 and 38 5 degrees since Lloyd and Wilson (1957) found the depression of the monosynaptic potential to be much less at a body tempererature of 34° than at 38° C (see also Kuno 1964 Klussman Stelter and Spaan 1969)

The comparison between the effects of dorsal root and peripheral nerve stimulation under various experimental conditions disclosed some differences However, it must be stated that the monosynaptic reflex recorded from a peripheral nerve in response to dorsal root stimulation is not strictly comparable with the one recorded from a ventral root in response to peripheral nerve stimulation The number of neurones excited by dorsal rot stimulation is not a function of a strict homosynaptic excitation as peripheral nerve stimulation in the adult animal since synergic afferents giving collaterals to this motoneurone pool are also excited When the monosynaptic tential is recorded from a ventral root in response to peripheral nerve stimulation eterosynaptic responses are added to the homosynaptic reflex in the immature animal as shown by Wilson (1962) (see also Eccles Shealy and Willis 1963) This is the case only after repetitive stimulation in the adult animal (*cf* Lloyd Hunt and McIntyre 1954) It can therefore be stated that with dorsal root stimulation there will be a mixture of homosynaptic and synergic excitation of the motoneurone pool under study Peripheral nerve stimulation will give homo and heterosynaptic activation in the young animal but only homosynaptic in the older and adult animal The monosynaptic responses studied in these experiments however have one set of neurones in common those activated by homosynaptic stimulation

When considering the different effects between peripheral nerve and dorsal root stimulation on the extensor monosynaptic potentials it therefore appears justified to conclude that dorsal root stimulation adds an initially excitatory component which could be synergic activation in the youngest kittens (Fig 5 A) In the older animal (Fig 5 C) the reverse is true that is dorsal root stimulation gives a larger initial depression than peripheral nerve stimulation However in both the young and the older kitten these differences tend to disappear after 60 sec stimulation (Fig 6 A and C) In the young animal the prolonged stimulation of a dorsal root reduces the initial excitation whereas in the older animal a prolonged stimulation of the dorsal

root gives further excitation Both the initial excitatory effect of dorsal root stimulation in the young animal and the later excitatory effect in the older animal are removed by Nembutal® This is in accordance with the observation of Hagbarth and Naess (1950), that polysynaptic activity is reduced by barbiturate anesthesia It is thus likely that this drug removes the synergic excitatory activation since it has a depressive effect on the monosynaptic transmission (Weakley 1969) This idea is further supported by the observation that initially tonic motoneurones showed phasic characteristics when Nembutal® had been given (Henneman, Somjen and Carpenter 1965 a)

Since the initial depression with increased stimulation frequency was found to be more pronounced on stimulation of a peripheral nerve than when stimulating dorsal roots in the young kittens the main cause of the initial depression must be sought in the monosynaptic path itself Skoglund (1960 b) concluded that dorsal root collaterals in the newborn and young animal are unable to carry high frequencies of discharge but become hyperpolarized even to the point of block However, the low frequency stimulation used here seems not to interfere with the initial ability of the afferents to excite the motoneurones The greater ease by which a monosynaptic reflex can be elicited in the young animal (*cf* Wilson 1962, Eccles Shealy and Willis 1963 Eccles and Willis 1963) seems to compensate more or less fully for a possible low frequency presynaptic depression However, in spite of the fact that the afferent fibres mature with increasing age and are able to carry higher frequencies of discharge, they obviously become less able to fire the motoneurones The increasing initial depression with increasing age is thus but a reflection of the development of a less safe reflex transmission The decrease of the extracellular concentration of potassium probably lowers the motoneurone excitability (Skoglund 1967) but the increase of the motoneurone size (Mellstrom and Skoglund 1969) may also be a contributing factor As shown by *e g* Henneman (1957) and Henneman Somjen and Carpenter (1965 a, b) it is easier to excite a small cell synaptically than a big one Last but not least a change in the motoneurone synaptology probably caused by the growth of the motoneurones (Conradi and Skoglund 1969) might lead to a change in the ability of the afferents to excite the motoneurones The percentage covering of boutons with flattened vesicles on the soma increases and those with spherical vesicles decreases on the motoneurone soma with increasing age of the kittens (Conradi and Skoglund 1969) This observation could under the prerequisite that the former are inhibitory and the latter excitatory (Uchizono 1965 1966) explain the decreased excitation and increased inhibition since many inhibitory actions are mainly concentrated on the soma according to Smith Wuerker and Frank (1967)

The flexor motoneurones seem to differ from those of the extensors not only with regard to the existence of a subliminal fringe in the newborn stage but the initial low frequency depression is also larger in flexors than in extensors in the younger stages and increases less than that of the extensors with age These observations favour the idea that the flexor afferents in the newborn stage might be more

mature than the extensor afferents (Skoglund 1960 b, Wilson 1962) Actually there is a difference of the maturation of their peripheral nerve fibres Thus the calibre spectra of the ankle flexor muscle nerves are more mature than those of the extensors (Mellstrom 1971 b) In the paranodal region of these nerve fibres the occurrence of degenerating myelin also speaks in favour of a more advanced developmental stage of the flexors (Berthold and Mellstrom to be published) These differences between flexor and extensor nerves have disappeared after the first month A more mature synaptological pattern in the flexor motoneurone pool might also be at hand explaining the presence of a subliminal fringe As found here in the adult cat the flexor monosynaptic reflex increases initially when increasing the stimulation frequency from 1 to 2/sec, but this does not occur with the extensor monosynaptic reflex (cf Fuortes and Hubel 1956 Beswick an Evanson 1957) The steady state depression of both the extensor and flexor are of the same order of magnitude in the adult stage some 15—20 % However the steady state depression of the extensor in the young animal is on the order of 30—50 %, whereas the corresponding depression of the flexor is much less This also indicates that the flexor monosynaptic pathway is more mature at birth

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From the Department of Anatomy, Karolinska Institutet, Stockholm, Sweden

Recurrent and Antidromic Effects on the Monosynaptic Reflex during Postnatal Development in the Cat

By

ANDERS MELLSTROM

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Abstract

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The recurrent effects on the amplitude of the ankle extensor and flexor monosynaptic reflexes were studied at different rates of stimulation during postnatal development in high spinal cats The recurrent + antidromic effects on the ankle extensor monosynaptic reflex were also investigated Recurrent inhibition of extensors was observed in the youngest kittens but disappeared more or less during the first postnatal month to reappear during the first part of the second month At that time, recurrent inhibition of flexors also appeared as did recurrent facilitation All recurrent effects were found to increase with stimulation frequency and with the exception of the extensors during the first month, they also increased in both magnitude and duration with age, reaching adult values during the second postnatal month It was concluded that the increased recurrent effects are partly due to a reduction of the excitability in the monosynaptic pathway, although a postnatal change in the recurrent effects per se can not be excluded Recurrent + antidromic effects on the extensors were also found to increase in magnitude and duration with increasing age

The aim of the present investigation was to study the recurrent and the antidromic + recurrent effects on the monosynaptic potential from ankle extensor and flexor nerves during postnatal development These experiments were undertaken not only to gain information about probable postnatal changes with age in the recurrent effects, but also as a means to study further the postnatally changing excitability in the monosynaptic pathways, as disclosed in a preceding publication (Mellstrom 1971 a)

In adult cats the recurrent and the antidromic + recurrent effects have been extensively studied since the investigations by Renshaw (1941, 1946) In spite of all the information gathered about the recurrent effects in the adult animal (*e g* Eccles Fatt and Koketsu 1954, Kuno 1959, Granit and Rutledge 1960 Wilson and Burgess 1962, Wilson, Talbot and Kato 1964) the role of this feedback circuit for the locomotor system is still under debate (see Eccles *et al* 1961 a, Wilson 1966)

Naka (1964 a, b), studing spinal cord function prenatally in the kitten, found a long lasting powerful recurrent inhibition at a gestation age of about 45 days, the

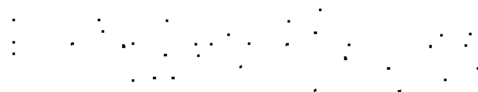
gestation time for cats being 65±4 days (Scott, Da Silva and Lloyd-Jacob 1957) Earlier authors (Skoglund 1960, Wilson 1962) studied these and antidromic+recurrent effects during the postnatal period but did not perform any systematic investigation of these developmental changes

It will be shown in this investigation that the recurrent effect on the monosynaptic reflex varies with the stimulus rate and increases with age The antidromic+recurrent effects also increase with age The recurrent effect on flexors differ from that on extensors during the first month in such a way that it supports the conclusion reached in an earleir publication (Mellstrom 1971 a), that the flexor monosynaptic pathway is more mature than that of the extensor at birth

Material and Methods

This investigation includes results from experiments on some 60 spinalized kittens from the newborn stage up to two and a half months as well as adult cats The operative procedure was mainly the same as discribed in a previous report (Mellström 1971 a)

The dorsal root L7 or S1 was stimulated supramaximally for the monosynaptic potential with square wave shocks of 0 3—0 5 msec duration through isolation transformers The monosynaptic potential was recorded from different ankle extensor and flexor muscle nerves These nerves were also antidromically stimulated through isolation transformers The stimulus strength was supramaximal for the A spike recorded on a more proximal part of the corresponding nerve since the frequency and duration of firing recorded from Renshaw cells is known to be positively correlated to the stimulus strength of the antidromic volley (*cf* Renshaw 1946 Eccles *et al* 1961 b) Each antidromic stimulus preceded the dorsal root stimulus by 1—100 msec and in some cases even more The recurrent effects on the monosynaptic potential (the



any long term variations in the amplitude of the monosynaptic potential are as far as possible excluded When the conditioned value differed more than 10 % from the control value it was considered a significant result (For further methodological details see Mellstrom 1971 a)

Results

A Recurrent effects on the monosynaptic reflex at different test frequencies

The recurrent effects on the monosynaptic reflex (expressed in percentage of the unconditioned control value) were found to vary in magnitude with the age of the kitten It must be emphasized that with the test rates used there were no residual effects from one test to subsequent ones

In Fig 1 the monosynaptic potential was elicited 1/9 sec from the dorsal root L7 and recorded from the plantaris nerve (extensor) This potential was conditioned by an antidromic supramaximal shock on the medial and lateral gastrocnemius nerves (extensors) The maximal inhibition thus obtained was hardly more than 10 % of the control value during the first postnatal week If however, the test rate was increased to 1/sec or 2/sec the unconditioned monosynaptic potential decreased by

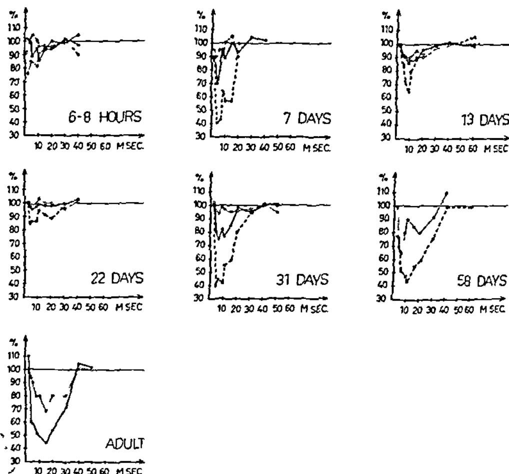


Fig 1 Recurrent effect on the relative amplitude of the monosynaptic potential (ordinate) versus interval in msec between conditioning and test stimuli (abscissa) Conditioning antidromic stimulus to the medical and lateral gastrocnemius nerves Test stimulus applied to the dorsal root L7 and recorded from the plantaris nerve In this figure and in Fig 2 3 and 6 each point is the mean of ten conditioned potentials The mean amplitude of ten unconditioned monosynaptic potentials equals 100 % Three different test rates were used 1/9 sec (o o) 1/sec (●———●) and 2/sec (o— ——— -o) In graph from the 58-day-old kitten (o — — — — —o) indicates a test 1/3 sec

some 30—50 % compared with that obtained at a stimulus rate of 1/9 sec (cf Mellstrom 1971 a) and there was an increase of the recurrent inhibition to about 30 % in the first case and 60 % in the latter (Fig 1) It is obvious that the recurrent effects become less during the second to fourth week of the first month (Fig 1), but an increase in test frequency still causes an enhancement After four weeks the recurrent effects again become pronounced increasing to about 60 % when the highest frequency was used However, from 58 days on, the recurrent effects at the slowest test rate (1/9 sec) have also increased to adult values The different stimulation frequencies have the same effect on the relative amount of recurrent

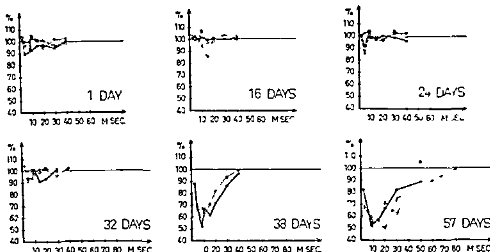


Fig 2 Recurrent effect on the relative amplitude of the monosynaptic potential (ordinate) versus interval in msec between conditioning and test stimulus (abscissa) Conditioning antidromic stimulus to the anterior tibial nerve Test stimulus applied to the dorsal root L7 and recorded from the long and short extensor digitorum nerves Three different test rates were used 1/9 sec (o o), 1/sec (● ——— ●) and 2/sec (o — — — — — o)

inhibition in the adult animal as in the newborn (Fig 1) Maximal recurrent inhibition was obtained in the adult cat when the conditioning stimulus preceded the dorsal root stimulus by 10—15 msec, whereas a somewhat shorter interval between the stimuli had to be used in the young kitten to obtain maximal effects probably due to differences in the conduction time in the peripheral nerves Further it appears (Fig 1) that the duration of the recurrent inhibition increases with increasing post natal age The adult values (Kuno 1959, Wilson Talbot and Diecke 1960) are reached in the second month

The monosynaptic reflex was also recorded from physiological ankle flexor muscle nerves, usually the short and long extensor digitorum nerves cf Wilson *et al* 1960) and sometimes from the anterior tibial nerve Generally the results from flexors have been somewhat more inconsistent than those from extensors and the small changes obtained more difficult to evaluate This is mainly due to the difficulty of obtaining stable flexor monosynaptic reflexes in the spinal preparation (Mellstrom 1971 a) The recurrent effects between two flexor nerves were studied by conditioning the anterior tibial nerve and recording the monosynaptic potential on the long and short extensor digitorum nerves or in some cases the reverse The recurrent inhibition between ankle flexors (Fig 2 was usually not obtained until after the first postnatal month but in one relatively mature animal as judged from the peripheral nerve conduction velocity it appeared 16 days after birth In the 38 day-old kitten (Fig 2) this inhibition was well established At this age the recurrent facilitation of from e

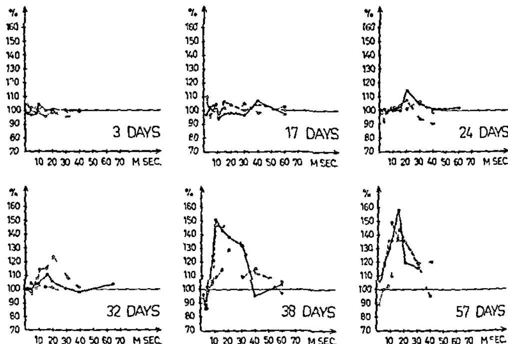


Fig 3 Recurrent effect on the relative amplitude of the monosynaptic potential (ordinate) versus interval in msec between conditioning and test stimulus (abscissa) Conditioning antidromic stimulus to the medial and lateral gastrocnemius nerves Test stimulus to the dorsal root L7 and recorded from the long and short extensor digitorum nerves Three different test rates were used *1/9 sec (o o) 1/sec* (•———•) and *2/sec* (o— — — —o)

The flexor monosynaptic potential in the long and short extensor digitorum nerves was conditioned by an antidromic stimulus to extensor muscle nerves the medial and lateral gastrocnemius During the first postnatal month no facilitation exceeding 10 % of the control value was as a rule seen neither at a test frequency of 1/9 sec nor of 1/sec (Fig 3) The oldest animal in which no certain recurrent *facilitation* was obtained at these two rates of stimulation was 32 days but at a stimulation frequency of 2/sec there was an unequivocal facilitation Whether a recurrent facilitatory affect was present in younger stages than 32 days was uncertain In a 38 day old kitten (the same as in Fig 2) however recurrent facilitation was found at all test rates used As is seen (Fig 3) the recurrent facilitation has a tendency to be more pronounced at the higher test rates especially in the animals about one month of age *With increasing age the elevation of the facilitatory effect increases* The early inhibition preceding the facilitation also becomes more accentuated with increasing postnatal age

The effects on the monosynaptic reflex recorded from an extensor muscle nerve after conditioning by an antidromic volley in a flexor muscle nerve have also been studied during postnatal development No certain effects have been found (neither facilitatory nor inhibitory) at any test rate used From this can be concluded that

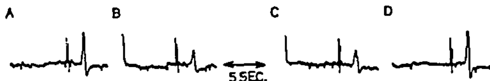


Fig 4 50-day-old kitten Dorsal roots L4—S4 severed In A the monosynaptic potential is elicited from the dorsal root L7 by a stimulus of supramaximal strength for the monosynaptic potential recorded from th
an antidromic stimulus tc
maximal for the A spike
effect In D a new control

the same situation as in the adult animal is present from birth on (*cf* Wilson *et al* 1960)

The recurrent inhibition of extensors is thus present in the youngest stages but this is not true for the flexors However, the extensor recurrent inhibition diminishes after the first week and then it increases during the second month at the same time as both recurrent inhibition and recurrent facilitation of flexors appear

The degree of recurrent inhibition was found to be related to both the age of the kitten and the test frequency used especially during the second month The increase in the recurrent inhibition of the monosynaptic potential with increasing test frequency has to be considered in the light of the change of the monosynaptic reflex with increasing stimulation frequency (*cf* Mellstrom 1971 a) The recurrent effect was found to be maximal from the first conditioned potential and did not change during subsequent stimulation (Fig 4). However, if the conditioning stimulus alone was given during some minutes a steady state of the inhibition was not reached when testing until some monosynaptic potentials had passed (Fig 5) This indicates that the first test stimuli are more effective in overcoming the recurrent inhibition This suggests that the relative amount of recurrent effects seen is a function of the excitability in the monosynaptic pathway (*cf* Granit and Rutledge 1960 Granit, Haase and Rutledge 1960)

B Antidromic+recurrent effects on the ankle monosynaptic reflex

The summed effects of antidromic and recurrent inhibition on the monosynaptic reflex recorded from the medial and lateral gastrocnemius nerves were studied during postnatal development Only one stimulation frequency (1/9 sec) was used

The antidromic+recurrent effects became more pronounced with increasing age (Fig 6) in agreement with the findings of both Skoglund (1960) and Wilson (1962) The duration of the recorded depression of the monosynaptic reflex is also seen to increase with age These findings might partly be explained by the postnatal incre‿e of the recurrent inhibition (Wilson 1962 and partly by the fact that the probabilit of antidromically invadir

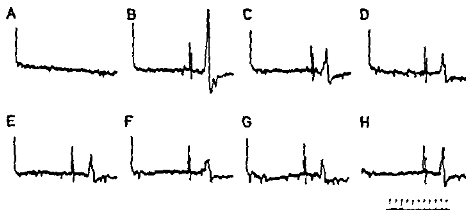


Fig 5 50-day-old kitten Dorsal roots L4—S4 severed A Recording from the plantaris nerve and antidromic supraximal stimulation (for the A-spike) of the medial and lateral gastrocnemius nerves B—G The dorsal root L7 is also stimulated supraximally for the monosynaptic potential recorded from the plantaris nerve Note the successive decrease of its amplitude In H the monosynaptic potential is recorded without any conditioning stimulus Test 1/sec Time in msec The curves are lightly retouched

Discussion

Both recurrent inhibition and facilitation were found to increase relatively with increasing stimulation rate The recurrent inhibition of ankle extensors was obtained in the youngest kittens but disappeared more or less during the first postnatal month It reappeared during the early second postnatal month when recurrent inhibition and facilitation of flexors first appeared Adult values of these effects were reached at the end of second month These results will be discussed in the light of earlier results with regard to postnatal excitability changes in the monosynaptic pathway (*cf* Mellstrom 1971 a)

The advantages of using an unanesthetized spinal animal immobilized with Flaxedil® were discussed in a preceding paper (Mellstrom 1971 a) It is generally agreed

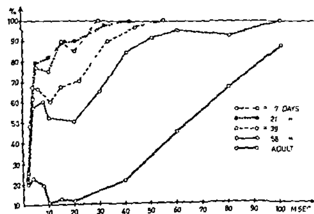


Fig 6 Recurrent + antidromic effects on the relative amplitude of the monosynaptic potential elicited from a dorsal root and recorded from the medial and lateral gastrocnemius nerves versus time in msec separating conditioning antidromic stimulus on the same peripheral nerve (abscissa) Test 1/9 sec

that acetylcholine is the transmitter between the ventral root collaterals and the Renshaw cells (*cf* Eccles 1969) and Flaxedil® is known to interfere with cholinergic transmission at least in muscle However, Eccles *et al* (1956) found no effect of Flexedil® on the firing frequency of Renshaw cells and this substance was also used by Wilson (1962) when studying recurrent inhibition in kittens

As earlier shown in the adult cat by Renshaw (1941) and Wilson *et al* (1960), it was now found, that also in kittens an antidromic stimulus to an ankle extensor muscle nerve exerts an inhibitory effect on the monosynaptic reflex from another extensor muscle nerve and a facilitatory effect on that of an antagonistic flexor nerve The recurrent effects from a flexor nerve inhibit the monosynaptic potential from another flexor nerve, whereas no recurrent effects are exerted from a flexor on the monosynaptic reflex from an extensor

In earlier experiments in kittens no attention has been paid to the fact that both the facilitatory and the inhibitory recurrent effects become relatively larger when increasing the stimulation frequency This is especially the case during the second postnatal month That an increased stimulation frequency should increase the recurrent effect per se is not compatible with the observation made by Renshaw (1946) that there is no increase in the firing frequency of the interneurones when conditioning an antidromic stimulus with another one on the same nerve Eccles *et al* (1961 b) showed however that the firing frequency could be affected by the strength of the antidromic stimulus If thus strength of the recurrent stimulation is made maximal it appears safe to assume that the amount of recurrent inflow imposed on the motoneurones is maximal at the stimulation frequencies used in this study This assumption is supported by the observation made here that a supramaximal antidromic stimulus gives a maximal recurrent effect from the first conditioned sweep on Consequently changes in the relative effect of recurrent activitation on a monosynaptic reflex when increasing the stimulation frequency ought to be due to changes in the monosynaptic pathway itself

With regard to the recurrent effects of extensors on extensors at the higher test rates (1/sec and 2/sec) there is a clear inhibition from birth on (*cf* Skoglund 1960) This increases with increasing stimulation frequency during the first week and then diminishes during the next fortnight to increase and reach adult values in the second month With regard to the effects of flexors on flexors there is no or a small inhibition from birth on during the first month and reaches adult values in the second month The effect of extensors on flexors gives no recurrent facilitation in the newborn stage but thus effect appears during the latter part of the first month and reaches adult values in the second month At all stages of development the effect increases with increasing stimulation frequency and this is most pronounced during the second month With the exception of the recurrent inhibition of extensors from extensors during the first month the effect also increases with increasing age The duration of the recurrent effects also seem to increase with increasing age (*cf* Wilson 1962) Skoglund (1960 found recurrent inhibition in the newborn stage to be less powerful but of twice the duration as in the adult stage This result is probably to

be explained by the fact that he used anesthetized animals (*cf* Mellstrom 1971 a) This explanation probably also holds true for the results of Skoglund (1960) obtained on antidromic+recurrent effects Thus he showed that the recovery of the mono synaptic potential from the gastronomius nerve after antidromic activation was of as long a duration in the newborn as in the adult cat Wilson (1962) found it to be of shorter duration as shown here and explained his finding as depending on the small or absent recurrent effects in the nerves he used whereas Skoglund s results are *probably due to a long lasting recurrent effect*

The above mentioned results should be considered in the light of the postnatal changes that occur in the monosynaptic pathway In an earlier publication (Mell strom 1971 a) it was stated that the initial depression of the monosynaptic potential increases with increasing age and increasing stimulation frequency due to a de creasing monosynaptic excitability and thus the gradual creation of a subliminal fringe

Since there is a recurrent inhibition of extensors in the newborn stage it is rea sonable to assume that recurrent effects are present from birth on which is also in agreement with the findings of Naka (1964 b) The reason why recurrent inhibition is obtained on extensors but not on flexors during the first postnatal week might be *that the extensor afferents are less mature than the flexors (cf Mellstrom 1971 a b)* and consequently they are less able to overcome the recurrent inhibition The re duction of the recurrent inhibition of the extensors during the second to fourth postnatal weeks might partly be due to the maturation of the extensor afferents reaching the same effectiveness as the flexors in overcoming the recurrent inhibition The reappearance of the recurrent inhibition on the extensors and its appearance on the flexors during the second postnatal month should then be due to a decrease in the ability of the afferents to overcome inhibition which is also indicated by the increase of the initial depression with increasing stimulation frequency (Mellstrom 1971 a) The appearance of the recurrent facilitation can only be explained by the *creation of a subliminal fringe which when subjected to disinhibition* (Wilson and Burgess 1962) gives an enhancement of the monosynaptic reflex

The relative increase of the recurrent effects seen with increasing stimulation fre quency might of course simply be due to the possibility that motoneurones having no recurrent inhibition fall within the subliminal fringe and consequently recurrent inhibition will increase proportionally (*cf* Granit Pascoe and Steg 1957) On the other hand the increased stimulation frequency might also diminish the mono synaptic excitation of the motoneurones and thus recurrent inhibition overcomes the excitation (*cf* Kuno 1959 Granit and Rutledge 1960 Granit Haase and Rutledge 1960) Those neurones on the other hand that are just about to be fired will do so under the influence of recurrent facilitation which also increases with increasing stimulation frequency

Of course a true postnatal development of the recurrent effects can not be ex cluded but at least part of the increasing recurrent effects seen with increasing age must be explained by the postnatally occurring excitability changes in the mono-synaptic pathway (*cf* Mellstrom 1971 a) A final settlement of the relative contribu

tion of these changes and of a real increase of recurrent affects in establishing the mature pattern might be achieved from intracellular studies

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From the Department of Pharmacology, Karolinska Institutet Stockholm, Sweden

Ca^{++} Dependence of Histamine Release and Formation of Slow Reacting Substance in the Cat Paw

By

KJELL STRANDBERG

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Abstract

STRANDBERG, K *Ca dependence of histamine release and formation of slow reacting substance in the cat paw* Acta physiol scand 1971 *82* 500—508

[illegible]

ethylene diamine tetraacetate (EDTA) In both instances the EDTA blockade could be reversed by Ca^{++} whereas Co Mg Mn and Ni were ineffective substitutes The EDTA blockade of histamine release but not that of the formation of SRS was also reversed by Sr and in some experiments by Ba The stimulatory effect of Sr^{++} on the release of histamine was weaker than that of Ca^{++} No significant stimulatory effect of Sr on the formation of SRS was observed It is concluded that the release of histamine and the formation of SRS in the cat paw are Ca^{++} dependent processes although in the case of histamine release Ca can partially be replaced by Sr

Certain polymer substances such as antigen and compound 48/80 release histamine from mast cells by activating energy requiring reactions (for references see Uvnäs 1964) However while anaphylactic histamine release has been shown to be a Ca^{++}-dependent process irrespective of the *in vitro* system studied (*cf* Humphrey and Jaques 1955 Mongar and Schild 1958 Chakravarty 1960, Lichtenstein and Osler 1964) it is not as clear that Ca is needed for the histamine releasing effect of compound 48/80 Thus histamine release from isolated rat peritoneal mast cells (Uvnäs and Thon 1961) or mixed rat peritoneal cells (Van Arsdel and Bray 1961) is virtually the same with or without Ca in the incubation medium whereas compound 48/80 induced histamine release from rat lung tissue (Chakravarty 1960, Rothschild 1970) or rat mesenteries (Mota and Ischii 1960) is depressed by Ca lack.

In some animal tissues histamine release induced by antigen and compound 48/80 has been shown to be accompanied by the formation of a smooth muscle stimulating principle termed 'slow reacting substance (SRS) (for references see Chakravarty 1959 b, Brocklehurst 1962) Ca lack has been shown to inhibit the release of

histamine as well as the appearance of SRS in anaphylactic reaction in guinea-pig lung tissue (Chakravarty and Uvnas 1960). Whether Ca^{++} is required for the formation of SRS evoked by compound 48/80 has not been studied

In this paper is reported the influence of Ca^{++} and other divalent cations on the histamine and SRS releasing effects of compound 48/80 in the cat paw.

Methods and Materials

Cat paw perfusion

The technique has been described in detail elsewhere (Strandberg 1971). The perfusion medium had
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Before mounting the paws were perfused, until free from blood, with a salt solution of the same composition as to be used in the experiment They were mounted in temperature con-

Assay of histamine

Histamine was determined fluorometrically (Shore, Burkhalter and Cohn 1959), using an Aminco-Bowman spectrophotofluorometer and 10 mm cuvettes The amines values quoted are expressed in terms of the free base

Assay of SRS

The amounts of SRS in the perfusates were determined by biological assay on the guinea pig ileum as described by Chakravarty (1959 a) The preparation was suspended in a 4 ml organ bath *containing aerated Tyrode solution (37° C) with atropine sulphate (1 μg/ml)* and mepyramine maleate (1 μg/ml) The volumes administered to the organ bath never exceeded 0 4 ml If no contraction was produced, the activity of the perfusate was considered as non-measurable SRS activity is expressed in SRS units referring to a standard of cat paw SRS (Strandberg 1971)

In the highest concentrations, Ba^{++} sometimes produced weak stimulatory effects When Co^{++}, Mn^{+} and Ni^{++} were used, the smooth muscle stimulating activity of the perfusates could not be determined directly due to interference with the biological assay Therefore the SRS

tion No corrections for losses during the extraction procedures were made The recovery of a SRS standard carried through the procedure is about 70 per cent (Strandberg and Uvnäs 1971)

Statistical analysis

Statistical analysis (Student s t test) was performed as described by Dixon and Massey (1957)

Results

Effect of Ca^{++}

Table I shows the effect of Ca^{++} on the efflux of histamine and SRS from cat paws induced by compound 48/80 It can be seen that Ca significantly stimulated both

TABLE I Effect of Ca^{++} on the release of histamine and the formation of SRS in the cat paw The amounts of histamine and SRS appearing in the effluents during perfusion with compound 48/80 (1 µg/ml) for 80 min are presented The spontaneous release is deducted The statistical calculation is based on the differences (d) in per cent between the yields from paws receiving no Ca^{++} (controls) and those from paws perfused with Ca^{++}

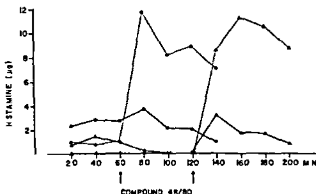
Exp no	mM Ca^{++}													
	0 (Control)		0 9						3 6					
	Histamine	SRS	Histamine			SRS			Histamine			SRS		
	µg	units	µg	% of control	d	units	% of control	d	µg	% of control	d	units	% of control	d
1	26 9	17,640	54 2	201	101	20 160	116	16	61 8	229	129	47,020	267	167
2	34 2	1,540	40 4	118	18	3,010	195	95	57 7	169	69	6,165	400	300
3	15 6	12,400	35 3	226	126	9,690	78	—22	50 2	322	222	12,830	103	3
4	4 7	8 840	7 3	155	55	10,590	120	20	12 5	266	166	6,200	70	—30
5	23 8	5,290	24 8	109	9	6,190	117	17	51 2	215	115	11,000	208	108
6	11 2	6,100	26 6	238	138	12,075	198	98	21 7	203	103	11,200	184	84
7	42 7	5,900	63 4	148	48	12,200	207	107	98 6	231	131	9,500	161	61
8	7 5	8,300	33 9	452	352	11,390	137	37	27 4	366	266	10 000	121	21
Mean					106			46			150			88
±SEM					39			17			23			37
p					$<0\ 05$			$<0\ 05$			$<0\ 001$			$<0\ 05$

the release of histamine (106—150 %) and the formation of SRS (46—88 %) as mpared to the outputs when Ca -free medium was used The stimulatory effect of Ca was apparent already at the lowest concentration used, 0 9 mM Ca had no leasing effects *per se*

Some histamine and SRS appeared after compound 48/80 even though Ca was omitted from the perfusion medium (Table I) Provided Ca was needed to activate both processes sufficient Ca must have been present in the tissue It was therefore studied (n = 4) whether the tissue Ca could be reduced by prolonged perfusion with Ca -free medium As shown in Fig 1 the releasing effect of compound 48/80 was depressed by perfusing the paws with Ca free medium alone for 1 or 2 hrs prior to the administration of the releasing agent The results thus indicate a common dependence on Ca for the compound 48/80 induced release of histamine and formation of SRS in the cat paw

Effect of EDTA

In an attempt to chelate endogenous Ca to achieve further evidence for a Ca^{++} dependence 2 mM EDTA was included in the perfusion medium in seven experiments (Fig 2) The control paws were perfused with Ca -free medium Compound 48/80 was added to the media after perfusion for 20 min and the perfusions were continued for 80 min EDTA was found to produce a significant reduction of the output of both histamine (77 %) and SRS (91 %)



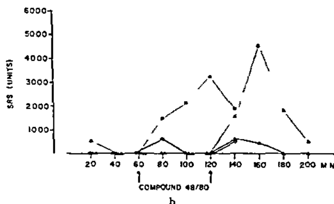


Fig 1 Effect of prolonged perfusion with Ca^{++} free medium prior to administration of compound 48/80 on the efflux of histamine (a) and SRS (b) from the cat paw Compound 48/80 was added to the perfusion medium when the paws had been perfused with Ca^{++} free medium for 1 hr, ○——○, or 2 hrs △——△ Compound 48/80 was at the same time also administered to the control paws (filled symbols) which were perfused with a medium containing 3 6 mM Ca^{++} throughout the experiment

Effect of other divalent cations

It was investigated, whether Ba^{+} and Sr , similarly to Ca could stimulate the release of histamine and the formation of SRS induced by compound 48/80 The results of four experiments are summarized in Fig 3 It is seen that 3 6 mM Sr^{++} increased the output of histamine as compared to the results when a medium devoid of divalent cations was employed However this concentration of Sr^{++} had no significant effect on the efflux of SRS Ba neither had a significant effect on the histamine release nor on the output of SRS As observed before Ca had a pronounced stimulatory effect on both processes As to the release of histamine the effect of Ca^{++} was about three times higher than that of Sr This indicates that Sr can only partially substitute for Ca in this process

Effect of divalent cations on EDTA blockade

The ability of Ba and Sr as well as of some other divalent cations, *i e* Co , Mg^{+}, Mn^{++} and Ni to reverse the inhibitory effect of EDTA on the release processes was studied and related to the effect of Ca^{++} Ca and to a lesser degree Sr were foun

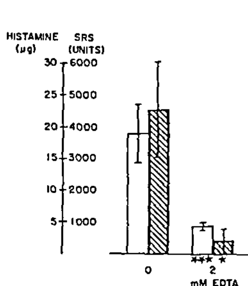


Fig 2

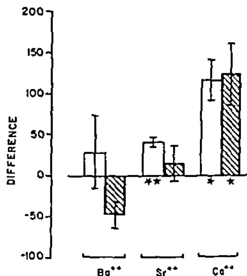
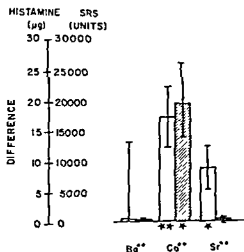


Fig 3

was based on the differences between the yields obtained from paws perfused with Ca++-free medium and those from paws perfused with EDTA

Fig 3 Effect of divalent cations on the release of histamine, □, and the formation of SRS, ▨, in the cat paw The paws, in a set, were perfused for 20 min either with Ca++-free medium or this medium to which 3 6 mM of Ba++, Ca++ or Sr++ had been added Then compound 48/80 (1 ml) was included and the perfusions were continued for 80 min The percentage differences between outputs of histamine and SRS from paws perfused with divalent cations and outputs from paws perfused without divalent cations are presented The estimated spontaneous release was deducted before calculation Means and standard errors of 4 expts, *, $p < 0.05$, **, $p < 0.01$



... the inhibi- ... compound ... to the media and the perfusions were continued for 80 min (II) The differences in output of histamine and SRS between the two periods (II—I) are presented Means and standard errors of 5—7 expts, *, $p < 0.05$, **, $p < 0.01$ The statistical hypothesis tested was II—I < 0

to reverse the inhibitory effect of EDTA on the histamine release (Fig 4) In two of the experiments (n = 5) with Ba^{++} this ion also proved to be effective In contrast, the EDTA produced inhibition of the formation of SRS was reversed only by Ca^{++} The other divalent ions (5 mM) tried (n = 3—5) had no effect on any of the processes

Discussion

The present results show that the release of histamine and the formation of SRS in the cat paw induced by compound 48/80 are Ca^{++}-dependent processes Thus both processes were stimulated by the addition of Ca^{++} to the perfusion medium and depressed by EDTA The inhibitory effect of EDTA was overcome by Ca^{++} However, there was one difference between the two release processes Thus, while Sr^{++} could partially substitute for Ca^{++} in the release of histamine, only Ca^{++} was effective when the formation of SRS was concerned This is interesting since the two processes are considered to be related events (Chakravarty *et al* 1959) However, it is possible that the release of histamine and the formation of SRS are triggered by a common mechanism activated by compound 48/80 and then the two processes proceed independently The effect of divalent cations on histamine release demonstrated in the present study is similar to that reported for anaphylactic histamine release where Sr^{++} can partially replace Ca^{++}, but Ba^{++} and Mg^{++} are ineffective substitutes (Lichtenstein and Osler 1964, Mongar 1970)

Ca^{++} has previously been shown to be needed in various processes leading to the release of biologically active principles from their intracellular granular storage, *e g* acetylcholine induced catecholamine release from cat adrenal chromaffin cells (Douglas and Rubin 1961, 1963, 1964), vasopressin release produced by electrical stimulation or excess K^{+} (Douglas 1963, Douglas and Poisner 1964) anaphylactic histamine release (Humphrey and Jaques 1955 Mongar and Schild 1958 Chakravarty 1960 Lichtenstein and Osler 1964) and release of enzymes from leucocytes evoked by stafylococcal leucocidin (Woodin and Wieneke 1963 1964) It has been proposed (Douglas 1968) that the function of Ca^{++} in this context is concerned with the delivery of the compounds to the cell exterior via exocytosis

The effect of Ca^{++} on compound 48/80 induced histamine release has also been studied but the results are somewhat contradictory Thus compound 48/80 induced histamine release from isolated rat peritoneal mast cells was about the same in the presence or absence of 1 mM Ca^{++} in the incubation fluid (Uvnas and Thon 1961) However, when a high concentration of compound 48/80 (10 μg/ml) was used a slight stimulatory effect of Ca^{++} was noted EDTA was ineffective in blocking histamine release both from mixed rat peritoneal cells (Van Arsdel and Bray 1961) and from isolated rat mast cells (Uvnas and Thon 1961) induced by the same releasing agent In contrast compound 48/80 evoked degranulation of rat mesentery mast cells (Hogberg and Uvnas 1960 Mota and Ischii 1960, Saeki 1964) histamine release from rat mesentery (Mota and Ischii 1960) and

(Chakravarty 1960 Rothschild 1970) were depressed by lack of Ca The present data add to the latter findings showing a requirement of Ca for the release of histamine from tissue mast cells The difference between the reactivity of mast cells *in situ* and that of isolated cells is puzzling and as yet unexplained

SRS has been suggested to be a split product resulting from the activation of a lytic enzyme, localized to the mast cell membrane during the release of histamine (Chakravarty *et al* 1959) There are some observations supporting this view Thus rat peritoneal mast cells have been shown to harbour an enzyme capable of hydrolysing lecithin (Keller 1962 Giacobini, Sedvall and Uvnas 1965) Furthermore in addition to SRS prostaglandin E_2 appears in the effluent from cat paws perfused with compound 48/80 (Änggård and Strandberg 1971) Prostaglandins are biosynthesized from precursor fatty acids split off from phospholipids (Lands and Samuelsson 1968 Vonkeman and Van Dorp 1968) and they appear on incubation or perfusion of various tissues with venom phospholipase A (Eliasson 1959 Vogt Suzuki and Babilli 1966) A similar endogenous enzyme has been suggested to be activated during the release of prostaglandins in connection with perfusion of frog intestine with salt solutions (Vogt *et al* 1966) and nerve stimulation (Coceani *et al* 1967)

Both prostaglandin like material and SRS were found when guinea pig lung (Fredholm and Strandberg 1969) and cat lung tissue (Ladinsky and Strandberg 1969) was incubated with bee venom phospholipase A The formation of these spasmogenic principles in guinea pig lung was reduced by EDTA (Fredholm and Strandberg unpublished This was probably due to chelation of Ca in the tissue ... e Ca^{++} is a co-factor of venom phospholipase A (for references see Condrea and de Vries 1965) and also of some mammalian phospholipase A preparations (Rimon Shapiro 1959 De Haas *et al* 1968 which cannot be replaced by other divalent cations Thus the present finding that Ca could not be substituted for any of the divalent cations tried when the formation of SRS was concerned seems to support the view (Chakravarty *et al* 1959 that the efflux of lipid-soluble spasmogenic principles from the cat paw might be due to the activation of an endogenous phospholipase A

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From the Department of Pharmacology, Karolinska Institutet, Stockholm Sweden

Efflux of Slow Reacting Substance (SRS) and Prostaglandins from Cat Paws Perfused with d-Tubocurarine and n-Octylamine

By

KJELL STRANDBERG

Received 1 February 1971

Abstract

STRANDBERG, K *Efflux of slow reacting substance (SRS) and prostaglandins from cat paws perfused with d tubocurarine and n octylamine* Acta physiol scand 1971 82 509—520

Release of histamine and formation of lipid soluble smooth muscle stimulating principles occurred when cat paws were perfused with d tubocurarine or n-octylamine Regardless of releaser the lipid soluble spasmogenic material was found to consist of two components one of [illegible] nciple with [illegible] lete loss of [illegible] ions of the [illegible] d chemical [illegible] The dose [illegible] induced by [illegible] ited by N ethylmaleimide potassium cyanide dinitrophenol and disodium ethylene diamine tetraacetate (EDTA) The results indicate that in the cat paw d tubocurarine and n-octylamine release histamine and induce the formation of identical or very similar lipid soluble spasmogenic principles by activating a common Ca dependent energy requiring mechanism

A substance producing a slow delayed contraction of the isolated guinea pig ileum has been reported to appear in the effluents from tissues perfused with snake venoms (Feldberg and Kellaway 1937 1938) and antigen Kellaway and Trethewie 1940 Brocklehurst 1960) Principles with similar smooth muscle stimulating activity termed slow reacting substance SRS) have been detected in the plasma of cat and dog after intravenous administration of the histamine liberator compound 48/80 (Paton 1951) and in the effluents from cat paws perfused with the same agent (Chakravarty Hogberg and Uvnas 1959 Anggård et al 1963 Strandberg 1971 a)

It has been suggested that the formation of SRS in the cat paw induced by compound 48/80 is linked to the activation of the histamine releasing process (Chakravarty et al 1959 Strandberg 1971 a) In the present investigation these

have been extended to other compounds known to release histamine in cat tissues, namely d-tubocurarine (Feldberg and Paton 1951) and n-octylamine (Feldberg and Mongar 1954)

Methods

Cat paw perfusion

The paws were taken from cats (≥1 5 kg) killed by injecting 1 g of sodium pentobarbital intraperitoneally [illegible]

dissol[illegible]

che[illegible]

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on the biological assay

Assay of histamine and SRS

Histamine

The fluorometric analysis described by Shore, Burkhalter and Cohn (1959) was followed using an Aminco-Bowman spectrophotofluorometer and 10 mm cuvettes The amine values given are expressed in terms of free base

SRS

The contents of SRS [illegible]

pig [illegible]

stan[illegible]

ml [illegible]

[illegible]

itrophenol could not be assayed directly due to interference with the biological assay In these experiments the effluents obtained after the introduction of compound 48/80 (4×20 [illegible] were combined and lyophilized as were the corresponding effluents from the control paws The lipid-soluble spasmogenic material was extracted with ethanol and ether and chromatographed on silicic acid *vide infra* The recovery in the extraction procedure is about 70 per cent Strandberg and Lynes 1971)

Purification and characterization of spasmogenic lipids

Preliminary purification

The effluents [illegible]

(100 μg/ml [illegible]

lyophilized p[illegible]

respectively [illegible]

three times with two volumes of ether at pH 3

Column chromatography

The combined ether extracts were subjected to sili[illegible]

100—200 mesh principally as described by Änggar[illegible]

elution system with methanol in chloroform M C [illegible]

ether fraction nonadsorbed material the residue [illegible]

(1 9) and applied to another silicic acid columr [illegible]

performed with increasing concentrations of ethyl [illegible]

(E B system) according to the scheme developed for the separation of prostaglandin E(PGE) compounds from prostaglandin F PGF compounds Samuelsson 1963)

Thin layer chromatography

Glass plates of dimensions 200 × 50 mm or 200 × 200 mm coated with Silica Gel H (E. Merck) approx. 0 25 mm thick were prepared and used as described in detail elsewhere (Strandberg

and Uvnäs 1971) The following systems were used, composition in ml, I Chloroform methanol acetic acid water (40 20 5 3) III Chloroform methanol water (60 35 8) A II Ethyl acetate · methanol acetic acid, 2,2,4 trimethylpentane water (110 20 30 10 100), the solvent mixture was equilibrated for 2 hours and the upper phase used (Gréen and Samuelsson 1964) When A II was used the adsorbent contained silver nitrate (1 g per 30 g silica gel) Reference prostaglandins PGE_1 and PGE_2 (10 µg), were visualized by spraying with 10 per cent ethanolic phosphomolybdenic acid and heating at 115° C for 15 min (Green and Samuelsson 1964)

Inactivation of biological activity

Spasmogenic material purified by extraction and silicic acid chromatography was used Samples were treated with phenyl isocyanate or iodine monobromide as previously described (Ambache 1959, Strandberg and Uvnäs 1971) The remaining biological activity was determined and expressed as a percentage of that of controls

Incubation of smooth muscle stimulating material with 0 2 mU of 15 hydroxy prostaglandin dehydrogenase isolated from swine lung (Änggård and Samuelsson 1966) was performed at 37° C for 30 min in 0 3 ml of 0 1 M Tris buffer pH 8 0, containing 5 mM NAD^+ Control incubations of the same material were performed where NAD^+ prostaglandin dehydrogenase or both were omitted from the incubation medium Directly after the incubation period the biological activity of the samples was determined

Smooth muscle preparations

The contractions of the preparations were recorded isotonically on a smoked drum using a frontal writing lever A dose cycle of 3 to 6 min with a contact time of 45—90 sec i e until the peak height was reached was used for all preparations

Guinea pig ileum

A segment of terminal ileum from guinea pigs weighing 200—400 g was suspended in a 4 ml organ bath containing aerated Tyrode solution with atropine sulphate (1 µg/ml) and mepyramine maleate (1 µg/ml) at 37° C

Guinea pig colon

A segment of proximal colon from guinea pigs, 200—400 g in weight, was suspended in a 4 ml organ bath Tyrode solution, 5 % CO_2 in O_2 37° C

Rat uterus

A segment of uterine horns from virgin rats weighing less than 100 g was suspended in a 0 5 ml organ bath containing de Jalon solution at 18—20° C gassed with 5 % CO_2 in O_2

Materials

Compound 48/80 was generously supplied by Dr B Hogberg AB Leo Helsingborg Sweden *d Tubocurarine* chloride was obtained from AB Vitrum Stockholm Sweden *n Octylamine* (puriss) was purchased as the base from Fluka AG, Buchs Switzerland The salt was prepared by the addition of conc HCl as described previously Feldberg and Mongar 1954)

Prostaglandin E_1 and prostaglandin E_2 were given by Professor S Bergström Dept of Chemistry Karolinska Institutet Stockholm Sweden 15 Hydroxy prostaglandin dehydrogenase was kindly supplied by Dr E Änggård this department

Results

Dose response relations

Cat paws were perfused with different concentrations of d tubocurarine (5—1000 µg/ml) and n-octylamine (1—500 µg/ml) for 80 min For comparison one paw within each set of paws was perfused with compound 48/80 (1 µg/ml) The results are illustrated in Fig 1 It is seen that, regardless of releaser, both the release of

have been extended to other compounds known to release histamine in cat tissues, namely d-tubocurarine (Feldberg and Paton 1951) and n-octylamine (Feldberg and Mongar 1954).

Methods

Cat paw perfusion

The paws were taken from cats (≥1.5 kg) killed by injecting 1 g of sodium pentobarbital intraperitoneally. Preparation, perfusion (1 ml/min at 27° C) and collection were performed as described [illegible] Strandberg 1971 [illegible]

[illegible]

chemicals were added in corresponding amounts to effluents from paws not perfused with inhibitor. This was performed to avoid the risk of a unilateral influence of the enzyme inhibitors on the biological assay.

Assay of histamine and SRS

Histamine

The fluorometric analysis described by Shore, Burkhalter and Cohn (1959) was followed using an Aminco-Bowman spectrophotofluorometer and 10 mm cuvettes. The amine values given are expressed in terms of free base.

SRS

The contents of SRS in the perfusates were determined by biological assay on isolated guinea pig ileum (Chakravarty 1959). SRS activity was expressed in SRS-units referring to a laboratory standard of cat paw SRS (Strandberg 1971 a). The volume administered to the bath was ≤0.4 ml. n-Octylamine was not present in the bath in concentrations higher than 10 µg/ml to avoid [illegible]pression of the reactivity of the guinea pig ileum (Mongar 1957). Perfusates containing [illegible]itrophenol could not be assayed directly due to interference with the biological assay. In [illegible]these experiments the effluents obtained after the introduction of compound 48/80 (4×20 min) were combined and lyophilized as were the corresponding effluents from the control paws. The lipid-soluble spasmogenic material was extracted with ethanol and ether and chromatographed on silicic acid (vide infra). The recovery in the extraction procedure is about 70 per cent (Strandberg and [illegible] 1971).

Purification and characterization of spasmogenic lipids

Preliminary purification

The effluents from paws perfused for 80 min with compound 48/80 (1 µg/ml), d-tubocurarine (100 µg/ml) and n-octylamine (100 µg/ml) were pooled respectively and lyophilized. The lyophilized perfusates were extracted twice with 80 per cent aqueous ethanol, 50 ml and 25 ml respectively. After evaporation of the ethanol content the resulting aqueous phase was extracted three times with two volumes of ether at pH 3.

Column chromatography

The combined ether extracts were subjected to silicic acid column chromatography (Unisil 100—200 mesh) principally as described by Änggård *et al.* (1963). A discontinuous gradient elution system with methanol in chloroform (M C system) was used. After evaporation of the ether fraction (nonadsorbed material) the residue was dissolved in ethyl acetate : benzene (1 : 9) and applied to another silicic acid column (Mallinckrodt 100 mesh). Elution was performed with increasing concentrations of ethyl acetate in benzene followed by methanol (E B system) according to the scheme developed for the separation of prostaglandin E (PGE) compounds from prostaglandin F (PGF) compounds (Samuelsson 1963).

Thin layer chromatography

Glass plates of dimensions 200 × 50 mm or 200 × 200 mm coated with Silica Gel H (E. Merck) approx. 0.25 mm thick were prepared and used as described in detail elsewhere (Strandberg

TABLE I Influence of enzyme inhibitors on the efflux of histamine and SRS from cat paws perfused with d tubocurarine (*100 µg/ml*) and *n-octylamine (100 µg/ml)* *The inhibitors were* present in the perfusion medium during the entire perfusion period (100 min) and the releasing agents were administered after the 20 min equilibration period Means and standard errors of 3 expts

Inhibitor molar conc	Inhibition in per cent of control			
	d tubocurarine		n-octylamine	
	histamine	SRS	histamine	SRS
N-ethylmaleimide 10^{-4}	17 ± 8 7	98 ± 1 7	49 ± 15	100 ± 0
Potassium cyanide 10^{-4}	69 ± 12	79 ± 16	79 ± 9 5	100 ± 0 4
Dinitrophenol 10^{-4}	75 ± 7 7	89 ± 9 4*	62 ± 17	88 ± 10*

* after silicic acid chromatography methanol chloroform (1 1) fraction

maleimide has been shown not only to antagonize histamine release induced by compound 48/80 but also to cause histamine release *per se* (Strandberg 1971 a) This fact must be taken into consideration when evaluating the present results

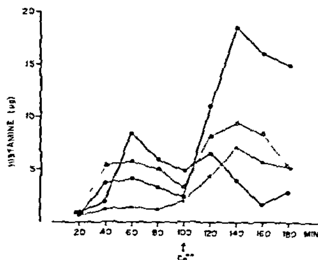
Influence of EDTA

The influence of EDTA on the release processes induced by d tubocurarine or n-octylamine was studied in three experiments Essentially the same effects were seen in all the experiments Fig 3 illustrates one of them It can be seen that there was an efflux of histamine and SRS when cat paws were perfused with d tubocurarine (100 µg/ml) or n-octylamine (100 µg/ml) dissolved in Ca free medium However these processes were depressed when 0 5 mM EDTA was administered before (20 min) and simultaneously with the liberator Subsequent introduction of 5 mM Ca resulted in an increase in the amounts of histamine and SRS appearing in the effluents The same amount of Ca was at the same time administered to the control paws which had not been exposed to EDTA This administration increased the output of both histamine and SRS in two of the experiments but not to the same extent as in paws treated with EDTA

Characterization of lipid soluble spasmogenic principles

Column chromatography

The non histamine spasmogenic material present in the effluents from paws perfused with d tubocurarine n octylamine or compound 48 80 elicited the same responses in an isolated guinea pig ileum preparation i e slow sustained contractions followed by slow relaxation (SRS response Fig 4 The lipid soluble smooth muscle stimulating material was extracted from the perfusates and subjected to silicic acid chromatography (M C system) in four experiments. This system effects separation of prostaglandins from SRS (Anggard and Strandberg 1971) Fig 5 gives the elution patterns from one such experiment as determined by biological assays on



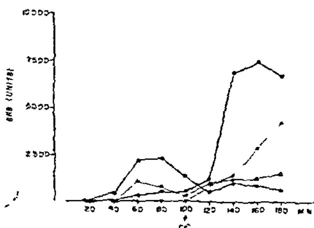


Fig. 3. Influence of EDTA on the release of histamine (A) and the formation of SRS (B) in the cat paw induced by d-tubocurarine (100 µg/ml) (circles) and n-octylamine (100 µg/ml) (triangles). The symbols represent the *amounts of histamine* and SRS in the effluents collected during 20-min periods. Releasers and chemicals were added to a Ca^{++}-free medium and administered as follows

	0—20 min	20—100 min	100—200 min
open symbols	0	releaser	releaser + 5 mM Ca^{++}
filled symbols	0.5 mM EDTA	releaser + 0.5 mM EDTA	releaser + 0.5 mM EDTA + 5 mM Ca^{++}



Fig. 4. Contractions of an isolated guinea-pig ileum produced by effluents from cat paws perfused with d-tubocurarine (DTC), n-octylamine (OCTA) and compound 48/80 (48/80) (4 ml bath, aerated Tyrode solution, 37° C, mepyramine maleate 1 µg/ml).

Fig 5 Silicic acid chromatography of lipid-soluble spasmogenic principles formed in cat paws perfused with d tubocurarine 100 μg/ml 2 cats n-octylamine 100 μg ml 4 cats and compound 48/80 (1 μg/ml) 2 cats Column 2 g M C system. Volume of the ether fraction 100 ml other fraction volumes 5 ml The biological activity was assayed on
a) guinea pig ileum. Activity expressed in terms of SRS units
b) rat uterus Activity expressed in terms of prostaglandin E- PGE

the guinea pig ileum and the rat uterus In all instances the biological activity was separated into two major constituents a less polar oxytocic component ether fractions) and a more polar component methanol chloroform 1 1 fractions the latter exhibiting the biological effects associated with SRS

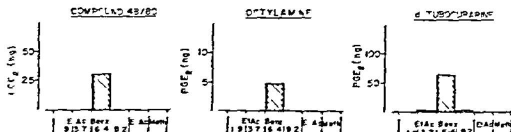


Fig. 6. Silicic acid chromatography of lipid-soluble spasmogenic principles (the ether fraction, Fig. 4) formed in cat paws perfused with d-tubocurarine, n-octylamine and compound 48/80. Column 1 g. E B system, fraction volume 25 ml. The biological activity was assayed on the rat uterus and expressed in terms of prostaglandin E (PGE).

The oxytocic component was rechromatographed on silicic acid using a less polar elution scheme (E B system) known to separate PGE compounds from PGF compounds (Samuelsson 1963). The result of one chromatography is shown in Fig. 6. It can be seen that, irrespective of releaser, the chromatograms showed mainly one biologically active peak (ethyl acetate: benzene 6:4) eluted at the expected position of a PGE compound.

Thin layer chromatography (TLC)

The similarity between the acidic lipid principles obtained by the use of the different releasing agents was further demonstrated on TLC. The results are summarized in Table II. Here it can be seen that the material present in the methanol: chloroform (1:1) fractions behaved almost identically in the two solvent systems used (I, III).

When the oxytocic material (ethyl acetate: benzene 6:4 fractions) was chromatographed against reference PGE and PGE (system A II) the biological activity was recovered from the zone corresponding to PGE.

Table II. Thin layer chromatography of acidic lipid principles purified by extraction and silicic acid chromatography. The Rf-values for the spasmogenic principles denote the zones from which biological activity was extracted. The Rf-values for PGE and PGE were established by spraying with 10 per cent ethanolic phosphomolybdenic acid and heating at 115° C.

System	Spasmogenic material	Bioassay preparation	Rf-value				
			d-tubocurarine	n-octylamine	compound 48/80		
I	Methanol chloroform (1:1)	guinea pig ileum	0.36—0.43	0.36—0.43	0.3[illegible]—0.43		
III	Methanol chloroform (1:1)	guinea pig ileum	[illegible]	0.21—0.29	0.21—0.3[illegible]		
A II	Ethyl acetate benzene 6:4	guinea pig ileum	[illegible]	0.36—0.4[illegible]	0.3[illegible]—0.4	0.[illegible]	0.4?

TABLE III Influence of chemical reagents on the biological activity of spasmogenic material purified by extraction and silicic acid chromatography (methanol chloroform 1 1) The results are expressed as percentages of the biological activity of controls Each value represents one experiment

Reagent	Releaser					
	d tubocurarine		n-octylamine		compound 48 80	
	Biological activity in per cent of control					
Phenyl isocyanate	0	0	0	0	0	15
Iodine monobromide	37	0	0	0	50	20

0 = non measurable

Inactivation of biological activity

Treatment of the spasmogenic material in the methanol chloroform (1 1) fractions with phenyl isocyanate or iodine monobromide resulted in a partial or complete loss of the biological activity (Table III) This indicated that the principle was dependent on hydroxylic group(s) and unsaturated bond(s) for its biological activity

The biological activity of the oxytocic material, purified by extraction and silicic acid chromatography, was completely abolished after incubation with 15 hydroxy prostaglandin dehydrogenase in the presence of NAD (Table IV) Control incubations where either the enzyme or the cofactor was omitted retained full activity Since the enzyme has been shown to be specific for the prostaglandins (Änggård and Samuelsson 1966) this finding demonstrates the prostaglandin nature of the spasmogenic principle

TABLE IV Inactivation of spasmogenic material with 15-hydroxy prostaglandin dehydrogenase (PGDH) The bioassay was performed on guinea pig colon

Releaser	Additions			Assay (ng of PGE_1)
	Perfusate extract	PGDH (0 2 mU)	NAD^+ (5 mM)	
d Tubocurarine	+	+	+	0
	+	—	+	17
n Octylamine	+	+	+	0
	+	—	+	13
Compound 48 80	+	+	+	0
	+	—	—	14
	+	+	—	12
	+	—	—	15
	—	+	+	0

0 = non measurable

Discussion

The mechanism of d tubocurarine induced histamine release from isolated rat peritoneal mast cells has recently been investigated (Frisk Holmberg and Uvnäs 1969) It was shown that the release process was rapid temperature- and pH-dependent and could be blocked by metabolic inhibitors features also characterizing the release process activated by compound 48/80 In contrast, the other agent used in the present study, n-octylamine and structurally related aliphatic amines are considered to de granulate rat mast cells and cause histamine release by a non-enzymatic mechanism, possibly by virtue of their high surface activity (Boréus 1960, Högberg and Uvnas 1960 Uvnäs 1961 Bloom and Haegermark 1967)

Evidence have been presented that formation of SRS in the cat paw does not occur in connection with non-enzymatic histamine release, *i.e.* cytolytic effects (Chakravarty *et al* 1959) It was therefore interesting at first that in the present study histamine release induced not only by d tubocurarine but also by n-octyl amine was accompanied by the formation of SRS However it was found, irrespective of releaser that both processes were inhibited by N-ethylmaleimide, potassium cyanide dinitrophenol and EDTA. The blocking effect of EDTA could be overcome by Ca^{++} These findings indicate that in the cat paw d tubocurarine and n-octyl amine, similarly to compound 48/80 (Strandberg 1971 a, b) induce the release of histamine and the formation of SRS by activating a Ca^{++}-dependent, energy-requiring mechanism. The results of the time course experiments further support a similarity between the mechanisms of action for the three releasing agents Moreover the results extend previous data suggesting a connection between the release of histamine and the formation of SRS in cat paw (Chakravarty *et al* 1959 Strandberg 1971 a, b

The comparative studies of the biological and chemical properties of the lipid soluble smooth muscle stimulating principles formed by the action of the different releasers showed that identical or closely related substances appeared in the effluents Thus regardless of releaser fractionation on silicic acid of the acidic lipid extract from the perfusates yielded two major spasmogenic components The component of less polar nature was identified as prostaglandin E_2 (PGE_2) by its chromatographic behaviour biological action and sensitivity to 15-hydroxy prostaglandin dehydrogenase. *The presence of PGE_2 in the effluents from cat paws perfused with compound* 48/80 has been reported earlier (Änggård and Strandberg 1971 The other *component elicited a typical SRS-response of the isolated guinea pig ileum Exposure to phenyl isocyanate and iodine monobromide abolished or caused a pronounced decrease in its biological activity indicating that intact hydroxyl group s and un saturated bond s are important for the spasmogenic activity of this principle*

The appearance of prostaglandins in the effluents from guinea pig lungs perfused with bee venom phospholipase A was recently reported Vogt *et al* 1969 Formation of lipid-soluble spasmogenic principles with characteristics similar to those reported on in the present study occurred when guinea pig lung tissue was incubated

with bee venom phospholipase A (Fredholm and Strandberg 1969) These findings are interesting in view of the fact that prostaglandins originate from phospholipid bound precursor acids (Lands and Samuelsson 1968, Vonkeman and Van Dorp 1968) Rat peritoneal mast cells have been shown to display phospholipase A like activity (*Keller 1962, Giacobini, Sedvall and Uvnas 1965*) Results suggestive of an increase in this activity were obtained in experiments where the cells were incubated with compound 48/80 (Giacobini *et al* 1965) If an endogenous phospholipase A is activated during the release processes in the cat paw, as originally suggested by Chakravarty *et al* (1959), split products of phospholipid hydrolysis might appear in the effluents The efflux of prostaglandin E_2 as well as of another lipid-soluble unsaturated hydroxy acid, *i e* SRS from cat paws perfused with histamine releasing agents is in accordance with this hypothesis

This investigation was supported by grants from Karolinska Institutet and the Swedish Medical Research Council (B 71 14 X 2943 03) The skilful technical assistance given by Mrs Birgitta Ask and Miss Marita Tingeborn is gratefully acknowledged

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FELDBERG W and J L MO [illegible] release [illegible] octylamine in peri [illegible] *Br J Pha ac* [illegible] 9 197 [illegible]

FELDBERG W and W D M PAT [illegible] Release of histamine from skin and [illegible] opium alkaloids a [illegible] he histamine liberat rs *J Physiol* [illegible]

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Acta physiol. scand. 1971. 82. 521—526

From the Department of Histology University of Lund, and Neurosurgical Clinic A, University Hospital of Lund, Sweden

Changes in Cerebral Blood Volume of Mice at Various Time-Periods after Superior Cervical Sympathectomy

By

L. Edvinsson, Ch. Owman and K. A. West

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Abstract

Edvinsson, L., Ch. Owman and K. A. West *Changes in cerebral blood volume of mice at various time-periods after superior cervical sympathectomy* Acta physiol scand. 1971 *82* 521—526

The relative blood volume in the brain of mice was estimated after distribution in the blood stream of intravenously administered ^{131}I labelled human serum albumin The alterations in the blood volume was studied at various time-periods after bilateral excision of the superior cervical ganglia The blood volume was found to remain unchanged during the first 6 hrs post operatively At 12 hrs it was reduced by 28 per cent, whereas after 24 hrs it had increased to a level 34 per cent above that in the unoperated control animals The volume then normalized at 4 days after sympathectomy and even tended to be subnormal after 2 weeks It is suggested that the initial reduction in blood volume is caused by vasoconstriction as a result of the leakage of noradrenaline from the vascular adrenergic nerve terminals starting a few hours after sympathectomy The resulting degeneration activation of the smooth muscle receptors is known to subside within about 24 hours at which time a vasodilatation was found to occur It is probable that the vascular tone then returned to the preoperative or an even more pronounced degree due to the progressive development of supersensitivity of the denervated vascular receptors to normally circulating catecholamines.

Fluorescence histochemical studies have revealed that the pial arterial system, particularly the main pial arteries at the base of the brain, receives an extensive adrenergic nerve supply (Nielsen and Owman 1967 Spoendlin and Lichtensteiger 1967, Donath 1968 Falck *et al* 1968 Ohgushi 1968 Kajikawa 1968 1969) It was shown in the cat that adrenergic nerve terminals accompanied pial arteries on the cerebral convexities with a diameter down to 15—20 μ (Nielsen and Owman 1967) Pial arteries radiating into the outer layers of the cortex are supplied by only a few adrenergic nerve fibres and in the deeper regions of the brain the intracerebral arteries are usually devoid of adrenergic innervation Falck *et al* 1965, Donath 1968) The pial veins receive only a scarce adrenergic nerve supply (Nielsen and Owman 1967) The formaldehyde induced noradrenaline fluorescence in all pial adrenergic nerve fibres disappears within one week after bilateral removal

superior cervical sympathetic ganglia (Falck *et al* 1965, Nielsen and Owman 1967, Ohgushi 1968, Kajikawa 1968, 1969)

It has been known since long that stimulation of the cervical sympathetic system usually produces a clearly notable constriction of the pial arteries and a slight reduction in the cerebral blood flow (Forbes and Wolff 1928, Ask-Upmark 1935, Bouckaert and Jourdan 1936, 1949, Forbes and Cobb 1939, Lubsen 1941, Schneider 1953, Browne *et al* 1955, Ingvar 1958, Bloor *et al* 1969, James *et al* 1969) The effect of cervical sympathectomy or blockade of the cervical sympathetic ganglia are contradictory some investigators have observed signs of a cerebral vasodilation while others have reported no significant effects (Talbott *et al* 1929, Leriche and Fontaine 1936, Forbes and Cobb 1938 Harmel *et al* 1949, Naffziger and Adams 1950, Schenkin *et al* 1950, 1951, Murakami and Ando 1955, and others) This has led to the widely accepted view that the sympathetic nervous system has no importance in the regulation of the cerebral vascular system However, two obvious reasons for these inconsitent findings can immediately be suggested One is that in some instances the interference with the sympathetic system has involved only the preganglionic nerves and in others the postganglionic ones Another reason is that the effects have been studied at greatly different time periods after the interference, and thus such mechanism as degeneration release of the transmitter and denervation supersensitivity of the vascular receptors have not been taken into account In order to evaluate these possibilities, changes in the brain vascular system (*i e* alterations in the cerebral blood volume) has been studied in mice at various time periods after removal of the superior cervical sympathetic ganglia

Material and Methods

The experiments were performed on 73 albino mice of either sex weighing about 25 g The animals were maintained on standard pellet food (SAN bolagen Sweden) and tap water *ad lib* Thirteen of the animals served as untreated controls Sympathectomy was performed in 30 animals by bilateral removal of the superior cervical ganglia under light ether anesthesia Determinations of blood volume were performed 6 12 24 hrs and 4 and 14 days after the operation Six animals were used in each of these groups Sham operation was performed in 30 etherized animals by exploring the neck region bilaterally without removing the ganglia Also these animals were killed for determination of cerebral blood volume at the above time intervals

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volume in the brain and the plasma volume in the reference tissue (intestine) which was selected because it has a blood volume similar to that of the brain (Everett *et al* 1956) but is not neuronally supplied by the cervical sympathetic system (Alm *et al* 1971) The result for each individual animal was expressed as cpm/g brain tisue divided by cpm/g small intestinal tissue

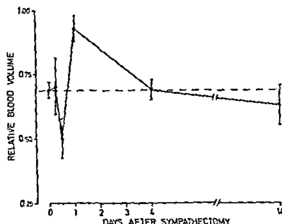


Fig 1 Relative blood volume of the brain (ratio between plasma volume of the brain and plasma volume of the reference tissue—small intestine—calculated from cpm per gram brain/cpm per gram intestine after intravenous injection of ^{131}I labelled albumin) before and after different periods of superior cervical sympathectomy The dashed line indicates control level of relative blood volume

Results

The alterations in the relative blood volume of the brain are evident from Fig 1 Six hrs after sympathectomy there was no change compared with the unoperated controls After 12 hrs the relative blood volume was reduced by 28 per cent (Student s *t* test $0.02 < P < 0.05$) Within the following 12 hrs the relative blood volume was almost doubled reaching a value that was 34 per cent above that of the non sympathectomized controls ($0.01 < P < 0.02$) The volume then returned to control values within 4 days postoperatively and 14 days after the operation it even showed a tendency to values lower than in the unoperated animals There was no statistically significant difference in the relative blood volume of the brain when comparing the sham operated animals with the unoperated controls

Discussion

The administered ^{131}I labelled serum albumin mixes with the plasma and does not penetrate through the blood brain barrier (*cf* Risberg *et al* 1969) The animals were killed by direct immersion into liquid nitrogen after injection of the albumin According to Stone (1938) a mouse is frozen solid in about 2 sec following immersion This rapid freezing technique circumvents erroneous results as a consequence of alterations in blood volume due to bleeding anesthesia method of killing the animal and due to shifts of blood resulting from manipulation of the animal during dissection Provided that the hematocrit value is not changed by the operation the radioactivity measured expresses the relative alterations in the blood volume after sympathectomy In order to correct for errors introduced by possible differences in the injected volume of the test solution the decay of ^{13} I and the disappearance of the albumin from the circulation the results were expressed as the individual ratio between the values of the brain and those of the small intestine which have been shown to have a blood volume comparable to that of the brain (Everett *et al* 1956) but which is not neuronally supplied by the superior cervical sympathetic ganglia (Alm *et al* 1971)

It is assumed that the values for the relative blood volume obtained pression primarily of the change in the diameter of those vessels k

with adrenergic sympathetic nerves i e the main pial arteries the arterial branches on the brain surface the proximal portion of the arterioles penetrating into the brain parenchyma and the pial veins (for references see Nielsen and Owman 1971) It is possible that the accompanying changes in the blood flow in these vessels will secondarily influence the flow (and the blood volume) in that part of the cerebro vascular system not innervated by sympathetic nerves

Although the functional significance of the neurogenic vasomotor control of the brain vessels is poorly understood at least the ability of the cervical sympathetic system to produce a vasoconstriction upon stimulation has previously been well demon strated Marked and varying changes in the relative blood volume of the brain were presently observed at varying time periods after bilateral excision of the superior cervical sympathetic ganglia For at least 6 hrs after the operation however relative blood volume remained unaltered which is in agreement with the findings that the noradrenaline content in sympathetically innervated organs remains virtually un changed during the first 8 hrs following sympathectomy (Weiner *et al* 1962 Ben miloud and Euler 1963)

Subsequently there is a leakage of the stored transmitter from the degenerating sympathetic nerve terminals (Malmfors and Sachs 1965 Van Orden *et al* 1967), which is accompanied by an activation of the effector structures (Sears and Barany 1960 Coats and Emmelin 1962 Langer 1966 Emmelin and Ohlin 1969 Lundberg 1969) With regard to the brain vessels this effector activation by the noradrenaline transmitter should be expected to result in a vasoconstriction which is in agreement with the present finding of a reduced relative cerebral blood volume 12 hrs after sympathectomy The receptor activation during degeneration of the sympathetic nerves continues for about 12 hrs (Lundberg 1969) after which the innervated smooth muscle relaxes The smooth muscle relaxation in the brain vascular walls results in a va odilation and concomitantly an increase in the relative blood volume of the brain as presently demonstrated

Four days after sympathectomy the relative blood volume of the brain had re turned to preoperative levels It has been shown in studies on the denervated fel ne nictitating membrane (Langer *et al* 1967) that the adrenergic receptors become sensitized to catecholamines by more than 100 times at this period after sympa thectomy The reduced relative blood volume can therefore be explained by a return of the cerebro vascular tone to normal levels as a response to the pre existing amount of catecholamines in the general circulation Furthermore it has been demonstrated (Langer *et al* 1967) that the denervation supersensitivity continues progressively and has reached almost 1000 times at 2 weeks after denervation It is therefore not unexpected that the relative blood volume in the brain shows a tendency to even subnormal values at this time period the circulating catecholamines will tend to *further increase the tone in the denervated brain vessels*

Principally the same findings have recently been reported for the sympathetic influence on ventricular fluid pressure in rabbits at various time periods after cervical sympathectomy (Edvinsson *et al* 1971 a and b) This offers further evidence in favour of the assumption that the registered changes in the ventricular fluid pressure

at least partly reflect alterations in the intracranial blood volume

It is obvious from the presented findings that interference with the cranial sympathetic nervous system will give a proper information about the sympathetic influence on the brain circulation only if the observations are made at an exactly defined point of time after the ganglionectomy (or ganglionic blockade) The observed changes in the cerebral blood volume cannot immediately be transformed in terms of alterations in cerebral blood flow However, they offer strong evidence in favour of the view that the sympathetic nervous system does influence the brain vessels in a way that can be expected for vasoconstrictor nerve fibres It has been shown in the present investigation that removal of the vasoconstrictor nerves to the brain vessels after a certain time period even can result in a vasoconstriction, a finding already made by Shackelford and Hegedus (1961) and previously considered unexpected

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Acta physiol scand 1971. 82. 527—531

From the Department of Histology, University of Lund and Neurosurgical Clinic A University Hospital of Lund Sweden

Alterations in Intracranial Pressure, Blood-Brain Barrier, and Brain Edema after Sub-Chronic Implantation of a Cannula into the Brain of Conscious Animals

By

L EDVINSSON, K C NIELSEN, CH OWMAN and K A WEST

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Abstract

EDVINSSON, L, K C NIELSEN, CH OWMAN and K A WEST *Alterations in intracranial pressure, blood brain barrier, and brain edema after sub chronic implantation of a cannula into the brain of conscious animals* Acta physiol scand 1971 82 527—531

recording was discontinued With trypan blue infusion only an initial transient and local

The findings are discussed in terms of the important error introduced by these pressure alterations when recording various functional parameters from electrodes needles cannulas and other small instruments implanted into the brain parenchyma

*Implantation into the brain of electrodes needles cannulas and other small instru*ments is a widely used technique for studies on a variety of physiological and pathophysiological reactions in the brain Besides the local injury caused by the instrument, the possibilities of more generalized changes within the cranial cavity is often neglected and the time course of the damage produced has received little attention Recently a cannula was devised for recording of the ventricular fluid pressure in conscious rabbits after induced intracranial hypertension (Owman and West 1…
In a further series of experiments (Edvinsson *et al* 1971), the effects on

tricular fluid pressure of surgical interference with the cranial sympathetic system was studied by continuous pressure recordings during up to 3—4 days In the course of these studies it became evident that the implantation of the recording cannula was in itself accompanied by marked alterations in the intracranial pressure The present report attempts to correlate the time-course of these pressure alterations with damage to the blood brain barrier and the development of cerebral edema

Methods

The *ventricular fluid pressure* was recorded in 10 conscious rabbits (2 3—3 kg b w) via a cannula (Owman and West 1970) inserted into the left lateral ventricle of the brain The outlet of the cannula was connected through a pressure sensing polyethylene catheter to a Statham model P23AC pressure transducer, the entire system being filled with physiological saline Recordings were performed on a Grass Model 7 Polygraph

The cannula was inserted into the brain of the conscious animal under local anesthesia (about 3 ml of 2 % lidocain, Xylocain Astra Sweden) through a burr hole placed in the fronto-parietal region according to the procedure previously given in detail (Owman and West 1970) The tip of the cannula itself was used for penetration of the dural and pial membranes The cannula was inserted into the brain by a slow, gentle and steady movement The right position of its tip in the ventricle was checked by the sudden appearance of the typical deflections due to respiration and arterial pulsations (Owman and West 1970) If such deflections

7 mm in length and 1 3 mm in diameter thus occupying 0 0093 cm³ volume The skin incision on the head was left open During registration the conscious animal was maintained sitting in a closed iron mesh box with its head and neck free The animal had free access to food and tap water during the entire experiment

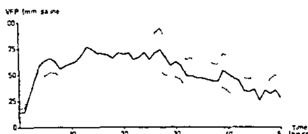
Any damage to the *blood brain barrier* was studied by trypan blue infusion (Hamberger and Hamberger 1966) The animals previously used for pressure recordings (see above) and 15 additional animals without pressure recordings (but with the cannula left in the brain for 1 hr 16 and 40 hrs) were anesthesized with Nembutal (1 8 ml i v to each animal of a 6 % solution diluted in physiological saline) A 1 % solution of trypan blue in physiological saline was infused into an ear vein (15 ml/kg b w) during about 5 min The common carotid artery was then freed and a polyethylene tube (1 5 mm outer diameter) was inserted with its tip in cranial direction and was held in position by a ligature About 10 min after completing the trypan blue infusion the jugular veins were opened and the vascular system of head was rinsed by perfus [illegible] animal died during this step [illegible] was not [illegible] carefully [illegible] After 3 [illegible] photogra[illegible] days formalin fixation and sectioned (6 μ) for fluorescence microscopy (Hamberger and Hamberger 1966) in order to obtain a more detailed information about any damage to the blood brain barrier (i e extravasation of trypan blue)

The extent of any *brain edema* and other microscopically visible changes were studied by light microscopy after staining in hematoxylin-eosin of alternate sections from the above mentioned preparations of the 25 animals

Results and Comments

The time-course of the alterations in the intracranial pressure (i e *ventricular fluid pressure*) produced by the implantation of the measuring cannula into the brain of otherwise normal, conscious animals is illustrated in Fig 1 The pressure measured immediately after introducing the cannula was 14 mm physiological saline Already

Fig 1 Continuous recordings of ventricular fluid pressure (VFP) during 50 hrs in 10 rabbits The pressure figure obtained for each time interval for each animal was the mean pressure calculated during a 1 hr recording The alterations in VFP (mean values ——, ± SE) are caused by damage (*cf* Fig 2) produced by the recording cannula itself, occupying an intracerebral volume of 0 0093 cm³



after 1 hr there was a fairly rapid increase in the pressure, after 4 hrs this had reached a level about 4 times higher than the initial pressure (Student's *t* test $0.01 < P < 0.05$) Within the following 1—2 hrs the pressure showed a further increase to approximately 5 times the initial pressure ($P < 0.01$) This level of ventricular fluid pressure (approximately 65—75 mm physiological saline) persisted during the following 25 hrs Between 30 and 35 hrs after implantation of the cannula the pressure showed a slow decrease, although 3 times higher than the initial pressure after 35 hrs, the increase was then not statistically significant ($P > 0.05$) This slow reduction continued progressively at 50 hrs after implantation of the cannula it was only twice the initial value ($P > 0.05$) In two animals the recording system remained patent for a further 25 hrs (not illustrated in the figure) in these two cases the pressure was found to remain at a steady level about twice that of the initial value It is possible that part of this relatively slight increase is caused by the intracranial volume occupied by the measuring cannula the increase being compensated for in the initial stage by a certain degree of hyperventilation The animals were all in good condition throughout the experimental period and the pressure level for any given time was constant provided the animals were left undisturbed The advantage in maintaining the animals in a fully conscious condition during the entire recording period is obvious from a physiological point of view It has been demonstrated that for example sodium pentobarbital increases the cerebrospinal fluid pressure in freely respirating dogs (Javid *et al* 1964) and rabbits (Edvinsson and West unpublished observations)

Iv infusion of trypan blue in attempts to visualize any damage to the *blood brain barrier* revealed only an initial transient and weak local reaction It has recently been shown that already placing the burr hole in the skull bone produces a circumscribed extravasation of the trypan blue within the surface of the gray matter corresponding to the diameter of the burr hole (Edvinsson and West 1971) As a result of the lesion in the brain parenchyma caused by the cannula a narrow zone of faint blue staining could be observed around the track Fluorescence microscopy (Hamberger and Hamberger 1966) confirmed that the extravasation of trypan blue

tricular fluid pressure of surgical interference with the cranial sympathetic system was studied by continuous pressure recordings during up to 3—4 days In the course of these studies it became evident that the implantation of the recording cannula was in itself accompanied by marked alterations in the intracranial pressure The present report attempts to correlate the time course of these pressure alterations with damage to the blood brain barrier and the development of cerebral edema

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were not directly obtained, the animal was discarded in order to avoid further traumatization of the brain during the manipulation A horizontal plane between the skull surface at the burr hole and the longitudinal central axis of the horizontally placed pressure transducer was chosen as zero reference plane The part of the cannula resting in the brain parenchyma measures 7 mm in length and 1 3 mm in diameter thus occupying 0 0093 cm^3 volume The skin incision on the head was left open During registration the conscious animal was maintained sitting in a closed iron mesh box with its head and neck free The animal had free access to food and tap water during the entire experiment

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cerebrospinal fluid absorption owing to the damage in the region near the sagittal sinus has promoted the intracranial hypertension It is conceivable that a long lasting application of trypan blue would have shown a more widespread staining of the brain but there is reason to believe (Davson 1967) that such a more extensive staining under the present conditions had revealed the extent of brain edema and not a blood brain barrier damage, since the staining after short lasting application was in fact only limited to the track area

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Acta physiol. scand. 1971. 82. 532—538

From the Department of Physiology, Karolinska Institutet, Stockholm, Sweden

Quantitative Evaluation of Release and Reuptake of Adrenergic Transmitter in the Rabbit Heart

By

ÅKE WENNMALM

Received 5 February 1971

Abstract

WENNMALM, Å. *Quantitative evaluation of release and reuptake of adrenergic transmitter in the rabbit heart.* Acta physiol. scand. 1971. *82*. 532—538.

Drugs known to block the reuptake of noradrenaline into sympathetic nerves were compared with respect to their potency to increase the outflow of noradrenaline from the isolated rabbit heart in response to nerve stimulation. At a dose level where the drugs tested strongly and equally blocked the uptake of exogenous noradrenaline, they appeared to form two distinct groups differing in potentiation of the noradrenaline outflow. Thus at optimal concentrations cocaine and desipramine were about equally efficient and increased the outflow of noradrenaline by about 100 %, while protriptyline, phenoxybenzamine and LU 3 010 raised the outflow by about 200 %. The effect of cocaine and desipramine on nerve stimulation induced outflow of noradrenaline was further increased by addition of the α blocking drug Hydergin. It is concluded that while all the drugs used increase the outflow of noradrenaline in response to nerve stimulation by strongly depressing reuptake of the transmitter into the neurons, PBA, protriptyline and LU 3 010 apparently in addition augment the amount of noradrenaline released from the nerves, possibly by counteracting some braking mechanism normally restricting the release of noradrenaline in response to nerve stimulation.

Perfusion methods are frequently used for quantitative estimation of the amount of noradrenaline (NA) released from sympathetic nerves in response to nerve stimulation. In such studies it is necessary to prevent the NA liberated from being trapped in the tissue, probably mainly in the nerves themselves (for review *cf.* Iversen 1967). Rebinding of NA in the nerves can be depressed either by using high frequencies of nerve stimulation, by adding drugs known to block uptake of NA into the neurons to the perfusion medium or by using high perfusion flow rates.

The drug most frequently used is phenoxybenzamine (PBA), which in addition to blocking the α-receptors and thus vasoconstriction also, at higher concentrations inhibits uptake of NA from the tissue fluids into the sympathetic neurons (Thoenen *et al.* 1964). In the presence of this drug it has been found that stimulation of the sympathetic nerves to the isolated cat spleen at frequencies of 10/s or less causes a strong increase in the outflow of NA from the organ. With this technique the amount of NA released by each nerve impulse in some experiments approaches the values

obtained when reuptake of the NA released is depressd by using high nerve stimulation frequencies (Brown 1965) The agreement between the results obtained when reuptake has been inhibited by drugs and by events accompanying depolarization of the nerves suggests that the estimates arrived at may give a reasonably good approximation of the true amounts of NA released from the nerve endings

However, the effects on nerve stimulation induced outflow of NA of drugs such as cocaine or imipramine, which have been reported to be much more efficient than PBA as inhibitors of neuronal uptake of NA, have been inconsistent, varying from no effect to a moderate increase (*cf* Iversen 1967) In view of this apparent discrepancy the present investigation was carried out to compare the effects of optimal concentrations of PBA on the nerve stimulation induced outflow of NA with those of some other uptake blockers The tissue chosen was the isolated perfused, sympathetically innervated rabbit heart, which has the advantage over *e g* the spleen that sympathetic nerve stimulation does not cause vasoconstriction

Material and methods

168 rabbits weighing from 1 2 to 2 4 kg were used for the study The animals were killed by a blow on the head and bled from the left carotid artery In experiments where sympathetic nerve stimulation was performed, the heart was dissected out with intact right and left sympathet[illegible]
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Na
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gauge transducer attached to the apex of the heart and using a Grass Model 5 Polygraph The perfusion pressure was kept at about 60 cm H_2O and the temperature at 37° C The electrodes for stimulation of the sympathetic nerves to the heart consisted of platinum rings in the wall of a tube through which the nerves and adjacent tissue were pulled The nerves were continuously superfused with Tyrode s solution The right and left nerves were stimulated simultaneously with 300 shocks of [illegible] delivered at 10/sec Each heart was stimulated [illegible] stimulation period which served as a control [illegible] the perfusion the second stimulation perio[illegible] respective

During the NA perfusion periods 0 5 ml aliquots from the perfusate taken 4 7 and 10 min after the beginning of the NA perfusion were counted in 20 ml of a 7 3 toluene absolute ethanol solution containing 4 g of 2 5 diphenyloxazole and 100 mg of 1-4 bis 2 (4 methyl 5 phenyloxazolyl) benzene per liter of toluene using an Intertechnique Abac SL 40 Liquid Scintillation Spectrometer

The drugs used [illegible] $\times 10^{-6}$ M) protriptyline (Concordin® M[illegible]
pthalane (LU 3-[illegible]
2×10^{-6} M) des[illegible]
mesylate (Hyderg[illegible]
to 1.2×10^{-6} M)

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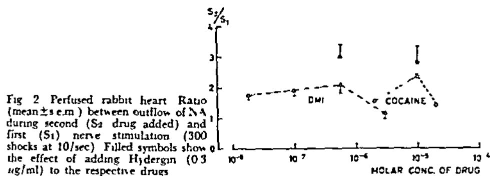


Fig 2 Perfused rabbit heart Ratio (mean $\pm$ s.e.m.) between outflow of NA during second (S_2 drug added) and first (S_1) nerve stimulation (300 shocks at 10/sec). Filled symbols show the effect of adding Hydergin (0.3 μg/ml) to the respective drugs

10^{-7} M) increased the nerve stimulation-induced outflow of NA by 133 ± 7 % and 106 ± 27 %, respectively (Fig 2). However the outflow of NA in response to nerve stimulation at optimal concentrations of PBA (3×10^{-6} M, 265 ± 45 %), protriptyline (10^{-8} M, 250 ± 10 %), or LU 3-010 (10^{-7} M, 199 ± 58 %) was significantly higher (Student's t-test, P = 2.82**, n = 22) than that in the presence of optimal concentrations of cocaine or desipramine (Fig 3). Hydergin (0.3 μg/ml) potentiated the effects of cocaine or desipramine on nerve stimulation induced outflow of NA, to 180 ± 52 % and 187 ± 3 % respectively, close to the range obtained for PBA, protriptyline and LU 3-010 (Fig 2). However, Hydergin, or the β-blocking drug pronethalol, did not significantly alter the effects of LU 3-010 on the outflow of NA in response to nerve stimulation. In fact, there was no significant difference in effect on the outflow of NA in response to nerve stimulation between PBA, protriptyline or LU 3-010 alone on the one hand, and cocaine or desipramine combined with Hydergin, on the other.

c Uptake of exogenous NA in the isolated rabbit heart

The completeness of the drug-induced block of removal of circulating exogenous NA, at the drug concentrations found to be optimal with respect to potentiation of NA outflow on nerve stimulation was tested at two concentrations of NA in the

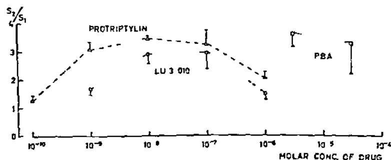


Fig 3 Perfused rabbit heart Ratio (mean $\pm$ s.e.m.) between outflow of NA during second (S_2 drug added) and first (S_1) nerve stimulation (300 shocks at 10/sec)

medium, 1 7 μg/l and 25 μg/l During perfusion with the lower NA concentration 85 ± 3 % (mean ± s e m) and with the higher NA concentration 86 ± 2 % of the NA infused appeared in the effluent from the heart When cocaine or desipramine (10^{-5} M and $5\ 5 \times 10^{-7}$ M) were added to the perfusion medium 99 ± 3 % of the radioactive NA appeared in the effluent Similarly, when PBA, protriptyline LU 010, or Hydergin together with cocaine or desipramine were added, 99 ± 1 % of the radioactive NA was recovered in the perfusate Thus, at the concentrations of NA used there was no difference in the completeness of the block of NA uptake between cocaine and desipramine on the one hand and PBA, protriptyline LU 3 010, or cocaine or desipramine together with Hydergin, on the other

Discussion

The marked prolongation of the chronotropic response to sympathetic nerve stimulation in the presence of optimal concentrations of uptake blocking drugs is in good agreement with earlier reports in the literature Thus potentiation of the response to sympathetic nerve stimulation has been demonstrated for cocaine in isolated rabbit atria (Hukovic 1959) and in the nictitating membrane (Haefely, Hurlimann and Thoenen 1964) Similar potentiations have also been observed with desipramine (Sigg Soffer and Gyermek 1963) PBA (Hukovic 1959) and LU 3 010 (Petersen *et al* 1966) These results are consistent with the view (Rosell Kopin and Axelrod 1963) that a major proportion of the adrenergic transmitter released by nerve stimulation is normally recaptured into the nerves and that reuptake plays an important role in the termination of action of the NA released from sympathetic nerves

Increased outflow of NA in response to sympathetic nerve stimulation after addition of uptake blocking drugs has previously been observed in the isolated rabbit heart where cocaine and desipramine more than doubled the outflow of NA on stimulation of the sympathetic nerve supply (Hukovic and Muscholl 1962 Loffelholz and Muscholl 1970) However in the present study optimal concentrations of PBA protriptyline or LU 3 010 caused significantly higher increase in the outflow of NA in response to nerve stimulation than cocaine or desipramine Both groups of drugs completely blocked the uptake of exogenous NA at the concentrations found to be optimal with respect to potentiation of nerve stimulation induced outflow of NA Thus it appears that PBA protriptyline and LU 3 010 in addition to blocking the reuptake of NA released also increase the amount of NA liberated Alternatively cocaine and desipramine might depress release as well as reuptake of NA Since some of these drugs have been shown to interfere with uptake or spontaneous release of NA from isolated nerve trunk vesicles (Euler and Lishajko 1968) part of the observed drug effects might be exerted on the intraneuronal storage particles However, the drug concentrations required *in vitro* were 10—10 000 times higher than those found to be optimal in the present study Thus it seems more probable that the level of action of the drugs is the secretory mechanism triggered by nerve stimulation The

finding that Hydergin added to cocaine or desipramine abolished the difference between the two groups of drugs in their ability to increase the nerve stimulation induced outflow of NA seems to favour the alternative of an increased release of NA by PBA, protriptyline and LU 3 010, rather than a depression of the same process by cocaine and desipramine

Recent studies in this laboratory (Wennmalm and Stjarne 1971) have strongly supported the hypothesis of an endogenous braking mechanism controlling the amount of NA released by depolarization of the sympathetic nerves (Hedqvist 1970) It is conceivable that PBA, protriptyline and LU 3 010 as well as Hydergin may prevent activation of the brake, and thus allow larger amounts of transmitter to be secreted each time the nerves are depolarized

The different uptake blocking drugs formed two distinct groups increasing the outflow of NA in response to nerve stimulation 2 2- and 3 4 fold respectively, compared to the preceding control stimulation It is at present not possible to decide which of these values is the best to unmask the true amount of NA released from the nerves However, in spite of this uncertainty the data suggest that normally, when the uptake mechanism is not depressed, at least one half of the amount of NA liberated, is recaptured into the neuron, even at the very high flow rates used in the present study Thus, reuptake of NA released is certainly important not only as a mechanism for termination of action of the NA released but by allowing its reuse also a convenient means to simplify transmitter economy (Stjarne 1964)

This work was supported by grants from the Swedish Medical Research Council (project B71 14X 3186 01) and from Stiftelsen Lars Hiertas Minne which are hereby gratefully acknowledged

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Acta physiol. scand. 1971. 82. 539—544

From the Department of Pharmacology, University of Göteborg, Sweden

The Effect of Protriptyline on the Disappearance and Nerve Impulse Induced Release of Labelled Metaraminol in the Rat

By

OLLE ALMGREN

Received 8 February 1971

Abstract

ALMGREN, O. *The effect of protriptyline on the disappearance and nerve impulse induced release of labelled metaraminol in the rat.* Acta physiol. scand. 1971. 82. 539—544.

The uptake and disappearance of ^{3}H-metaraminol (^{3}H-MA) after an i.v. injection of 40

After exogenous administration both noradrenaline (NA) and metaraminol (MA) are efficiently accumulated in the nerve terminals of sympathetically innervated organs (*Muscholl 1960, Hertting, Axelrod, Kopin and Whitby 1961, Andén 1964*, Shore, Busfield and Alpers 1964). MA differs from NA in being resistant to the catabolizing enzymes and also in being more lipid soluble (see Carlsson 1966). The disappearance of labelled NA from tissues has been subject of many studies with conflicting results (see Iversen 1967), although it is clear that the nerve impulse flow is of importance (Fischer and Snyder 1965). The disappearance of MA, which is considerably slower, is also, at least during the first 24 hours, dependent on the impulse flow of the sympathetic nerves (Almgren and Waldeck 1967).

It was felt that a more detailed information of different factors influencing the ^{3}H-MA disappearance could be of value for the understanding of the release process. In the present work the influence of the amine reuptake mechanism on the disappearance of ^{3}H-MA was investigated.

Protriptyline (PTP), a tricyclic thymoleptic drug, is well known to efficiently block *this amine concentrating mechanism at the nerve cell membrane* (Carlsson and Waldeck 1965) The effect of this drug on ^{3}H-MA disappearance was studied both in intact organs in decentralized organs, deprived of their physiological nerve impulse flow, and in organs where the parenchymal tissue was atrophied thus largely eliminating extraneuronal accumulation of the amine

Methods

Male Sprague Dawley rats were used About 14 days before the experiment the excretory ducts of the submaxillary and sublingual glands were ligated unilaterally in one group of rats After this procedure the glandular cells will atrophy (Junqueira 1951 Standish and Shafer 1957) In some of these rats and also in another group a preganglionic sympathetic denervation (de centralization) was performed about 7 days before the experiment The operations were per formed under pentobarbital (Nembutal Abbott) anesthesia In this way it was poss ble in the experiment to follow the uptake and disappearance of the amine in intact atrophied decentralized and atrophied plus decentralized salivary glands as well as in the intact heart

On the day before the experiment the rats were placed in a room with a constant tem perature of 29° C and were kept there until killing in order to prevent hypothermia

40 μg/kg *dl* metaraminol 7 ^{3}H (specific activity 1 63 C/mmole New England Nuclear Chemicals was injected in a tail vein Half of the rats were killed after 30 min 3 hrs and 6 hrs To the other rats protriptyline chloride in a dose of 10 mg/kg was administered i p 30 min after the MA injection Half of these rats were killed after 2 5 hrs i e 3 hrs after the administration of MA The remaining rats received an additional dose of 5 mg/kg PTP i p 3 hrs after the MA injection and were killed 3 hrs later The submaxillary plus sublingual glands and the hearts were taken out weighed and homogenized in ice cold 0 4 N perchloric acid After filtration the extracts were mixed with equal amounts of Packard Insta gel Emul sifier (Packard Instrument Company Inc and shaken to form an emulsion The total ^{3}H content was measured in a *Packard Tri Carb Liquid Scintillation Counter The efficiency* in this system is very constantly near 10 per cent In some con [illegible]
the ^{3}H MA content was measured with liquid [illegible]
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Results

Uptake of ^{3}H MA in salivary glands and heart

The content of ^{3}H MA in the differently treated glands 30 min after the i v in jection of 40 μg/kg of the amine is presented in Fig 1 It may be noted that de centralization did not significantly affect the uptake of ^{3}H MA After glandular atrophy the weight of the glands is reduced to about 1/3 The uptake of ^{3}H MA was reduced to approximately the same extent when calculat d per gland ($P < 0\ 001$) The uptake into the intact gland calculated per g tissue was 142 4 + 15 49 ng/g (n = 4) while the content of the heart amounted to 94 6 + 5 96 ng/g (n = 4) 30 min after the ^{3}H MA injection

Disappearance of ^{3}H MA from salivary glands and heart and the effect of PTP

In Fig 2 the levels of ^{3}H MA 3 and 6 hrs after the administration of the amine are presented in per cent of the amine content 30 min after the injection At the 3 hr interval there was a significant reduction of the amine level in the atrophied gland

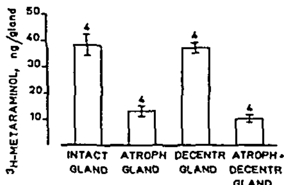


Fig 1 The content of ^{3}H MA in ng/gland 30 min after an i v injection of 40 μg/kg in intact, atrophied decentralized, and atrophied plus decentralized salivary glands of the rat The values represent means ± S E M

only ($P < 0.05$) After 6 hrs there was a significant reduction both in the intact and the atrophied glands ($P < 0.005$), but not in the decentralized or atrophied plus decentralized glands In the heart there was no significant change of the ^{3}H-NA level *during the time period studied*

After PTP treatment the ^{3}H-MA disapperance was accelerated in all glands and in the heart in all cases ($P < 0.001$ at the 6 hrs interval) This was most pronounced in the glands atrophied and decentralized At 6 hrs only about 12 per cent (totally 1 20 ng/gland) of the ^{3}H-MA content at 30 min was left, compared to about 25 per cent (totally 9 96 ng/gland) in the intact gland ($P < 0.001$) Also in the glands only atrophied or only decentralized the effect of PTP was more pronounced than in the intact glands ($P < 0.001$ and 0.005 respectively at the 6 hr interval)

When the amount of ^{3}H-MA found in the different tissues 5 5 hrs after the PTP administration was expressed as a percentage of the amount found at the cor-

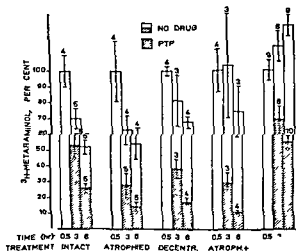


Fig 2 The content of ^{3}H MA in hearts (ng/g) and intact atrophied decentralized and atrophied plus decentralized salivary glands (ng/gland) of the rat 30 min 3 hrs and 6 hrs after i v injection of 40 μg/kg of the amine *10 mg/kg of protriptyline PTP)* was given i p 30 min after the ^{3}H MA injection and an additional dose of 5 mg kg 3 hrs after the administration of ^{3}H MA The values are expressed as a percentage of the amount of ^{3}H MA found 30 min after the injection of the amine (for absolute values see Fig 1 and text and represent means ± S E M The figures represent the number of experiments For tests of significance see text

responding time interval in animals not treated with PTP the following results were obtained heart 42 2±3 17 per cent, intact gland 46 0±4 81 per cent, atrophied gland 31 5±2 81 per cent, decentralized gland 24 8±1 93 per cent and atrophied plus decentralized gland 15 8±1 63 per cent The number of experiments are the same as given in Fig 2 Using an analysis of variance it was found that significantly smaller amounts of ^{3}H-MA remained in atrophied ($P < 0.025$) or in decentralized glands ($P < 0.001$) compared to intact glands In glands both atrophied and de centralized the effect was even more pronounced than in glands only atrophied or decentralized ($P < 0.025$)

Discussion

The uptake of ^{3}H-MA into the intact submaxillary gland calculated per g tissue, was slightly higher than the uptake into the heart The distribution of ^{3}H NA after a single i v injection is different Here about 3 times as much of the labelled amine is found in the heart as in the submaxillary gland This has been explained by differencies in blood flow, since the uptake of ^{3}H NA *in vitro* in these two organs is directly correlated to the approximately equal endogenous NA levels (Almgren and Jonason 1971 a)

The reason for this difference between NA and MA is not known with certainty Possibly MA due to its metabolic stability remains in the blood for a longer time period than NA after a single i v injection The amount of amine taken up might then more reflect the concentration of adrenergic terminals in the organs than the blood flow through them

A relatively large extraneuronal binding of MA in the salivary glands could be another possible explanation In an earlier experiment (Almgren and Waldeck 1967) where a four times lower dose of MA was used the extraneuronal binding measured in denervated salivary glands amounted to about 2 ng/g compared to a total uptake of 8 ng/g in intact glands If the dose-uptake relationship for the extra neuronal tissue in this dose range is linear and independent of the neuronal uptake still at most 8 ng of the 38 ng found in intact glands after 30 min in the present study could be accounted for by extraneuronal accumulation This cannot explain the difference in uptake between NA and MA

The lower uptake of ^{3}H MA into the atrophied gland about 35 per cent of normal, has been observed earlier (Almgren and Waldeck 1967) Also the uptake of other amines is reduced of ^{3}H NA to about 20 per cent (Almgren and Jonason, to be published) and of labelled tyramine to about 15 per cent of normal (Almgren Anden and Waldeck 1965) The reduction of the endogenous NA content of the atrophied glands is small compared to the weight loss (Anden Norberg and Olson 1966) On the basis of these data is has been proposed that extraneuronal binding might influence the neuronal uptake of the transmitter (Almgren Anden and Waldeck 1965) A reduction of the blood flow of the atrophied glands must, however, also be considered

In this work the disapperance of ^{3}H-MA was studied during the initial phase, when the amine probably still is in positions from where it can be easily released (*cf* Crout *et al* 1964) Abolishment of the nerve impulse flow, accomplished by decentralization reduces the rate of disappearance (Almgren and Waldeck 1967) The present data are in agreement with the earlier observations

The disappearance of ^{3}H-MA from the atrophied gland did not differ from that of the intact gland After decentralization of the atrophied gland, however, there was no longer any significant decrease of ^{3}H MA These data indicate that there is still an impulse flow in the adrenergic nerves of atrophied glands Also, when the adrenergic nerves of atrophied glands are stimulated electrically ^{3}H NA or ^{3}H MA previously taken up is released to about the same extent as from intact glands (Alm gren, unpublished data)

When PTP was given to the rats after ^{3}H-MA administration, the disappearance of this amine was markedly accelerated also in the heart This effect of the membrane pump blockers on ^{3}H MA disappearance has been demonstrated earlier (Carlsson and Waldeck 1966) The disappearance of ^{3}H-NA, on the other hand, seems to be very little, if at all, affected (Titus *et al* 1966 Almgren, unpublished data) The difference between the two amines in this respect is probably due to the higher lipid solution and lower affinity for the granular storage mechanism of MA (Carlsson 1966) These two factors may allow higher extragranular concentra tions and a greater leakage of this amine out to the extraneuronal space Under normal conditions an appreciable part of the MA leaking out will probably be recaptured by the membrane pump Inhibition of this reuptake will reveal the leakage

The fact that the effect of PTP was more pronounced in the atrophied glands than in the intact ones might possibly be explained by the loss of extraneuronal binding sites Normally, in intact glands with a functioning membrane pump mechanism extraneuronal binding appears to be of little importance but will reach a significant level when the membrane pump is blocked *e g* by PTP (Almgren and Jonason 1971 b) In the virtual absence of extraneuronal binding sites as in the atrophied glands, more ^{3}H MA will thus disappear after inhibition of the membrane pump

Also in the decentralized glands there was an increased effec of PTP In these glands, deprived of the nerve impulse flow the concentrations of ^{3}H MA reached extraneuronally in the synapses are probably lower than in intact glands This might lead to smaller extraneuronal accumulation of the amine also in the decentralized glands following PTP treatment and consequently to an enhanced disappearance rate

Other posible explanations could be offered to explain the greater effect of PTP on decentralized glands than on intact ones It might be that a greater part of the labelled amine is localized outside the granules in decentralized than in intact glands resulting in a greater leakage This fits in with the fact that the most pronounced effect of PTP was seen in glands both atrophied and decentralized The hypothetical change in intraneuronal distribution of ^{3}H MA proposed above to occur after de centralization might be explained by a higher degree of saturation of gran

sites with NA, leading to displacement of ^{3}H-MA into the cytoplasmic sap

Anyhow, the fact that the PTP-induced loss of ^{3}H-MA was more pronounced after decentralization supports the view, that in this case we are largely dealing with passive leakage

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Acta physiol. scand 1971. 82. 545—554

From the Department of Physiology, Royal Veterinary College, Stockholm Sweden

Transducer Characteristics of the Muscle Spindle as Revealed by its Receptor Potential

By

D Ottoson and G M Shepherd

Received 9 February 1971

Abstract

Ottoson, D and G M Shepherd *Transducer characteristics of the muscle spindle as revealed by its receptor potential* Acta physiol scand 1971 *82* 545—554

The transducer properties of the sensory endings of the muscle spindle have been studied in isolated spindles of the frog's toe muscle The depolarization of the sensory endings during [illegible]

elastic elements take up the load or that the sensory membrane saturates beyond a given amount of distortion

After release of stretch the depolarization of the endings decreases exponentially with time The time course of repolarization is considerably slower than the return of the spindle to resting length and relatively independent of the rate of release of stretch This suggests that the repolarization is governed by the transduction process and related to the electrical proper ties of the sensory membrane

The muscle spindle may be regarded as a transducer which converts the deformation of the sensory endings into potential changes by which information is signalled to the central nervous system about the velocity and magnitude of length changes of the muscle (*cf* Katz 1950 1961 P B C Matthews 1964 Shepherd and Ottoson 1965) In studies of the function of the spindle the impulse response has generally been used as an index of the transducer output (*cf* Houk Cornew and Stark 1966, Toyama 1966) On the basis of these studies several models have been proposed to explain the transduction process However the first detectable electrical sign of the activity of the spindle is the generator current (Katz 1950 Ottoson and Shepherd 1965) For an understanding of the transducer mechanism precise knowledge is therefore required about the potential changes in the sensory endings Such a knowl edge is also essential for the development of a functional model of the spindle

The aim of the present study has been to analyze the mechano electrical ducing function of the spindle and the relation between the receptor curre

by the endings and the impulse discharge Special attention has been given to the time characteristics of the different phases of the electrical response

The results suggest that there is relatively little distortion of the dynamic properties of the stimulus in the course of its transmission to the endings It is likely that elastic elements in the spindle play an essential role in this process It appears further that the functional properties of the spindle cannot be explained by simple mechanical models employing elastic elements in series with viscous elements

Methods

The experiments were performed on isolated frog muscle spindles Isolation and recording was carried out as described in detail in earlier papers (Ottoson 1965, Ottoson and Shepherd 1965) Calomel half-cell electrodes connected to the preparation through Ringer agar bridges were used for the recording of the spindle response One electrode held the afferent nerve lifted up into paraffin oil and the other was placed in the Ringer solution surrounding the preparation The spindle was tied at one end to a fine rod fastened to the coil of a loudspeaker Stretches were applied by driving the loudspeaker coil with electrical pulses of different wave forms To obtain the receptor potential in isolation the conducted activity was blocked with 0 15—0 18 % lidocaine

Results

Receptor currents during dynamic and static stretch

The quantitative relationships between the amount and velocity of stretch and the changes in amplitude of the response have been described earlier (Ottoson and Shepherd 1965) In the present study attention has been focused on some specific features of the receptor currents pertinent to the problem of the relation between structure and function of the spindle

The superimposed records in Fig 1 show the typical changes in the rising phase of the receptor potential with changes in velocity of stretch With all rates of stretch

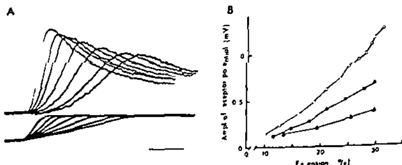


Fig 1 *A*, characteristic features of receptor ... al records of responses to stretches at different velocities ... monitor in this and following figures Time ... spindle and amplitude of receptor potential ... Amount of stretch given in per cent of resting length

ing the potential closely followed the extension of the spindle This was an invariable finding in all spindles when the stretch was moderate and extension of the spindle did not exceed 20—30 % of resting length One important feature of the responses was that there was no initial fast rising phase of the dynamic response as might be expected if the spindle behaved like a simple mechanical model with viscous and elastic elements in series (*cf* Houk *et al* 1966, Toyama 1966 Gottlieb Agarwal and Stark 1969)

It may be noted in Fig 1 (*A*) that there was a slight rounding of the peak of the response This was found to be largely if not entirely due to the retardation in time course of elongation in passing from the dynamic to static phase of stretch The amount of rounding of the stretch can be judged from the stretch monitor in Fig 1 *A* Correction was made for this rounding by plotting the response amplitude against the amount of stretch throughout the dynamic period Such plots (Fig 1 *B*) show a very nearly rectilinear relation between receptor current and stretch beginning soon after the onset of stretch and carrying through to the termination of the dynamic phase The rounding of the dynamic peak can therefore be regarded as reflecting the high rate sensitivity of the spindle to changes in velocity of the applied stretch

It will be noticed that even with the correction for the non linearities of the applied stretch the onset of the response was still slightly non linear in relation to the initial lengthening of the spindle The delayed onset of the response appears most likely to be due to some degree of slack that has to be taken up before lengthening becomes fully effective

In the experiments described above the spindle was not stretched by more than 30 % of its resting length When stretch went beyond this value the behaviour of the receptor potential was different and the rectilinear relation between lengthening and response magnitude no longer obtained This is illustrated by the records in Fig 2 In *a* the spindle was stretched by about 25 % of its resting length The response exhibited the typical increase in height with dynamic stretch and the ensuing decay to the static level during maintained stretch When the spindle was extended still more (*b*) the dynamic response did not increase further but remained at a relatively constant height forming thereby a saturation phase At the termination of the dynamic phase of stretch the response decayed from this plateau to a steady state level With still greater extension (*c*) the duration of the saturation phase increased but its level remained the same In some spindles there was a slight increase of the plateau while in others it decreased somewhat It may be noted that the static phase increased proportionally with increasing amount of stretch beyond 30 % of resting length With extreme extension of the spindle (100—150 %) the static phase therefore reached the same height as the saturation level For different velocities of stretching the saturation plateau was usually reached at about the same level of stretch

It has been demonstrated earlier (Shepherd and Ottoson 1965) that the frequency of the impulse response of the spindle increases in direct relation to the length of the spindle This relation does not hold however when the spindle is ex

A



B

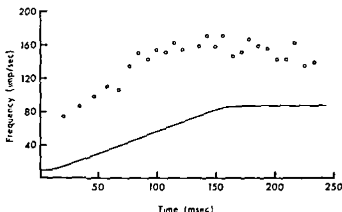


Fig 2 *A*, effect of overstretch Receptor potentials obtained by stretching the spindle to 25 % (a), 45 % (*b*) and 60 % (*c*) of its resting length Time bar 25 msec *B*, impulse response with strong, slowly rising stretch Note that impulse frequency levels off before completion of dynamic stretch

more than 30 % This is illustrated by the diagram in Fig 2 *B* which shows the frequency of impulse discharge during a slowly increasing extension up to 40 % of the resting length of the spindle The frequency of the discharge increased in direct relation to the stretch up to an extension of about 25 % of the resting length Beyond this value the frequency diagram levels off for the remaining part of the period of dynamic stretching This phase of relatively constant frequency of impulse discharge corresponds to the plateau of the receptor potential as illustrated in Fig 2 *A*

When extended and held at a constant length the receptor potential decays toward a quasi-steady level The time course of the initial rapid phase of the fall is illustrated by the superimposed records in Fig 3 which show the responses obtained with step stretches of different magnitudes The responses to extensions up to about 25 % of the resting length of the spindle show the monotonic decay from the dynamic peak to a steady level that has already been described At greater amounts of extension, however, the response departs from this pattern As can be seen in Fig 3 the dynamic response is followed by an "undershoot" in relation to the later static phase, and in some spindles there was a tendency to oscillatory behaviour before the response stabilized at the static level

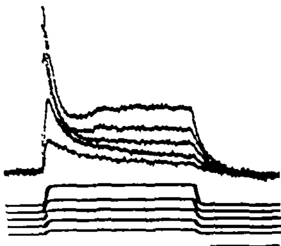


Fig 3 Changes in decay of receptor potential to static level with increasing amounts of stretch Superimposed records of response to steplike stretches Time bar 50 msec Lower trace shifted upwards for each recording to enable reading of stretch amplitudes resting length of spindle thus the same in all recordings

Decay of response following release of stretch

As described above, the magnitude and time characteristics of the dynamic stretch are closely reproduced by the rising phase of the response In contrast to this the time of the fall of the response after release of stretch follows a time course which differs significantly from that of the release itself This is illustrated by the recordings in Fig 4 *A* in which the responses to brief, purely phasic stretches of different strengths have been superimposed It can be seen that the potential decayed towards baseline with a monotonic time course which was considerably more prolonged than the release of stretch as indicated by the stretch monitor The values for the decays when plotted semilogarithmically versus time (*B* in Fig 4) fall on approximately straight lines and the slope of these lines becomes slightly steeper with increasing amount

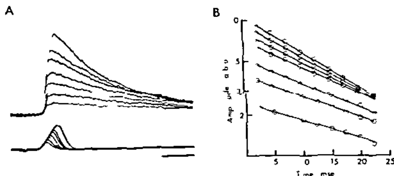


Fig 4 *A* decay of receptor potential following release of purely phasic stretches of different strengths Time bar 10 msec *B* time course of decay of responses in *A* Semilogarithmic scales



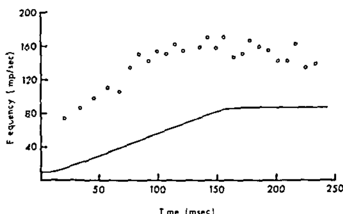


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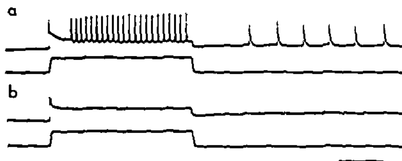


Fig 7 Afterdischarge following release of strong stretch (*a*) Record *b* shows the isolated receptor potential to the same stretch as in *a* Time bar 50 msec

When extension went beyond 50—75 % of resting length the response sometimes did not return to baseline but remained at a high level for several sconds This finding seems to explain why there sometimes was a persistent longlasting discharge after overstretches An example of this is illustrated in Fig 7 The spindle was subjected to a steplike extension to about 70 % of resting length This stretch produced a response with an initial dynamic spike and a pause which preceded the ensuing steady discharge Following release of stretch there appeared a longlasting afterdischarge The conducted activity was then blocked with lignocaine and the same stretch applied again At release of stretch the potential fell but did not return to baseline until after several seconds It is most likely that the after discharge of the spindle may be due to this remaining depolarization of the endings It should be emphasized that the spindle usually exhibited signs of being injured after such strong stretches It is reasonable to assume therefore that the longlasting depolarization may be attributed to mechanical damage of the spindle

Discussion

The present results show that the depolarization of the sensory endings during dynamic stretch of the spindle is a smooth process which closely follows the amount and rate of lengthening of the spindle This is not what would be expected if the spindle behaved like a simple model with viscous and elastic elements arranged in series (*cf* P B C Matthews 1964 Houk *et al* 1966 Toyama 1966 Gottlieb *et al* 1969) The behaviour of the spindle rather corresponds to that of an elastic element, and this has been verified by photomicrographs taken of the spindle during the dynamic phase of stretch (Ottoson and Shepherd 1968 1970 *a* The smooth rise of the response would also be provided by a system with elastic and viscous elements in parallel The gross viscous properties of the spindle are limited in magnitude, however, for the photographic studies (Ottoson and Shepherd 1970 *a*) show that the spindle returns quickly to rest following an applied stretch indicating a dominant elastic behaviour

A rectilinear relation between stretch and transducer action is valid for stretches up to a given level representing a lengthening of the spindle by about 30 % Beyond this value the receptor current levels off and a saturation phase of relatively constant depolarization develops the potential may show a slight increase or even a decrease The level of stretch at which the plateau phase was attained appeared to be approximately the same for different velocities of stretch The duration of the plateau phase for a given amount of stretch therefore is a function of the duration of stretching The development of the constant level of depolarization explains why the impulse frequency does not increase when stretch goes beyond 30 % of the resting length of the spindle It is the transducer elements and not the nerve fibre which fail to signal stronger dynamic stretching We wish to emphasize that it is not likely that the spindle *in situ* is subjected to stretches of such magnitude It may therefore be concluded that under physiological conditions the spindle response faithfully signals the dynamic lengthening of the spindle

The development of the plateau phase may be related to the mechanical properties of the spindle or the characteristics of the transduction process in the sensory terminals With regard to mechanical properties there is evidence that the elastic elements in the spindle do not lengthen linearly with applied load From tension measurements Husmark and Ottoson (1970) concluded that as stretch carries to higher levels lengthening of the elastic elements does not increase proportionally with force This mechanical property could provide the basis for the plateau phase of the dynamic receptor potential observed in the present experiments if sensory transduction occurs rimarily in relation to the extension of the spindle It is also possible that the rate ensitive mechanism of the sensory membrane responsible for the dynamic response aturates beyond a given level of extension This would be consistent with the finding that the plateau phase tended to appear at approximately the same level of extension with stretches at different rates Further experiments are needed to assess these factors

In discussion of the relation between structural and functional properties of the spindle relatively little attention has been paid to the period of return of the spindle to resting length after the release of stretch As shown it takes 30—50 msec for the response to return to baseline after release of stretch This is in marked contrast to the rapid return of the pulling rod to zero position (within a few msecs) Photomicroscopic observations have verified that the spindle itself also returns quickly to resting length (Ottoson and Shepherd 1970 *a*) There is therefore a dissociation of mechanical and electrical events during the period following release of stretch In addition the present results show that the decay of the response usually tends to be somewhat faster as the preceding stretch is made stronger or more prolonged In contrast the spindle under these conditions tends not to return quite as quickly to its resting position (Ottoson and Shepherd 1970 *a*)

These differences between mechanical and electrical events in the aftermath of stretch suggest that if the decay of the receptor current has a basis in mechanical movement that movement must be at the ultrastructural level involving the

mechanical environment of the sensory membrane Alternatively, the sensory membrane may return quickly to resting condition, as does the spindle grossly, and the prolonged potential decay could be determined by electrical properties of the sensory innervation (Ottoson and Shepherd 1970 *a*) The prolonged decay might imply a relatively long membrane time constant and relatively large membrane capacitance Unfortunately we have no independent evidence as yet for the latter quantity We would like to emphasize that an adequate interpretation of the decay of the current would require a reconstruction of the sensory terminals, the distribution of sites of transduction, and the relation of the extracellular recording electrodes to the pathways for current flow, for which the rigorous methods developed by Rall (1964) would be appropriate There is at present insufficient evidence to enable a complete reconstruction However, it may be noted that the present finding of a monotonic exponential decay of the receptor current following a brief stretch would indicate that most of the receptor terminals are involved in transducing the depolarizing response, and that the equivalent cylinder (*cf* Rall 1964) of the nerve branches through which the receptor current must spread to the recording site on the nerve, has a relatively long characteristic length This is consistent with previous evidence that the branches have a long characteristic length (Ottoson and Shepherd 1969) and that there is a relatively uniform depolarization of sensory terminals throughout the terminal chains (Ottoson and Shepherd 1970 *b*)

In conclusion, the characteristics of the dynamic response suggest that the elastic elements of the spindle play a dominant role in transmitting the stimulus to the endings It also appears that the dynamic properties of the stimulus are transmitted to the sensory endings with little distortion The viscous elements of the spindle appear to affect the dynamic transducer properties of the spindle relatively little The precise coupling of the elastic and viscous elements cannot be determined at present As pointed out by Katz (1961), more has to be known about the actual stress strain relations within different regions of the spindle before a definite conclusion can be drawn as to the relation between structural features and the transducer characteristics of the spindle Besides these mechanical factors we have noted that the sensitivity of the spindle to a dynamic stimulus is a property shared by other non mechanical sensory receptors (*i e* olfactory receptors Ottoson 1956 visual photoreceptors Fuortes and Hodgkin 1964 suggesting together with other evidence Ottoson and Shepherd 1968 1970 *a* *b*) that the dynamic sensitivity of the spindle could be largely a property of the transduction mechanism in the sensory membrane itself This possibility must now be considered seriously in addition to the more obvious mechanical factors Knowledge of all these factors is obviously required before an adequate model of the spindle can be proposed

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From the Department of Pharmacology, Karolinska Institutet Stockholm Sweden and the Department of Physiology, University of Pennsylvania Medical School, Philadelphia USA

Influence of Changes in Microcirculation on the Excitability of the Sensory Unit in the Tooth of the Cat

By

Lennart Edwall and Donald Scott Jr

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Abstract

Edwall, L and D Scott Jr *Influence of changes in microcirculation on the excitability of the sensory unit in the tooth of the cat* Acta physiol scand 1971 82 555—566

Simultaneous determinations of radioactive iodide disappearance rate (k value) and sensory nerve impulse frequency from dentinal cavities were performed on canine teeth of anesthetized cats Changes in k value reflecting changes in pulpal microcirculation were obtained by direct sympathetic nerve stimulation or by thermal stimulation Sympathetic nerve stimulation reduced the k value and induced an initial increase in nerve impulse frequency During maximal sympathetic nerve stimulation this initial increase was followed by a marked depression in impulse frequency Application of heat increased k value as well as impulse frequency while cold reduced k value and induced an initial increase in nerve impulse frequency followed by a decrease Thermal stimulation superimposed during maximal sympathetic stimulation when k value was depressed, was inadequate to evoke an appreciable increase in sensory nerve impulse frequency It is suggested that the excitability of sensory units in the tooth is strongly modulated by changes in pulpal microcirculation induced by for example stimulation of sympathetic vasoconstrictor fibres

Hypersensitivity to pain of human dental pulp has been shown to be correlated with morphological changes in the vascular bed indicating a change in the blood supply in the pulp (Grossman 1970) Neural activity recorded from electrodes placed in dentinal cavities in the canine tooth of the cat has shown both local and conducted potentials in response to thermal mechanical and chemical stimulation (Scott 1966) Increased tooth temperature was accompanied by an increase in single unit impulse frequency, the constancy of which was quantitatively dependent on environmental stability (Scott and Stewart 1965)

The structure of a sensory unit in dentin has been described by Frank (1966) and Arwill (1967) who have shown a receptor like structure associated with a fine afferent axon Electronmicrographs showed the presence of abundant mitochondria in the sensory unit In view of the presence of these inclusions an influence of the circulation on the excitability of the sensory unit would be a reasona

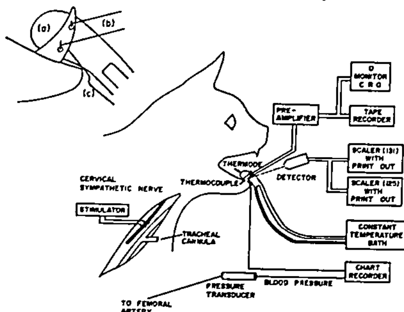


Fig 1 Drawing showing experimental set up Inset represents enlargement of tooth, thermode (a), electrodes (b) in cavities, thermocouple circuit (c) with thermosensible end (not seen in drawing) located close to cavity over pulp horn in contact with enamel and thermode For further explanation see text

pectation Therefore it seemed highly desirable to determine the extent to which changes in the local blood circulation in the pulp may produce predictable modifications of excitability of the sensory unit

Methods

Experiments were conducted on 28 cats, weighing 2 8—5 3 kg, anesthetized with sodium pentobarbital (30 mg/kg i v X-ray pictures were taken of the four canine teeth to determine the form of the pulp and the probable maturity of the dentin A tracheal cannula was inserted and provision made for recording femoral blood pressure Fixation of the head was provided by means of a steel rod inserted between the jaws and secured in place by dental acrylic, care being taken that the canine teeth remained fully exposed for cavity preparation and for insertion of a water circulated thermode to insure maintenance of constant temperature of the tooth under study throughout the experiment (Fig 1) The temperature of the thermode was controlled to 0 1° C and the temperature of the tooth [illegible]
to the recording cavity to insure tha [illegible]
alter the intended temperature This [illegible]
ture when thermal stimuli were applie [illegible]
from a second constant temperature bath

Based on the information from the X ray film cavity positions were chosen, one over the pulp horn and one within the gingival half of the crown At these locations the enamel was removed using a diamond instrument operated at slow speed Warm Macrodex® (Pharmacia) solution was used to prevent drying of dentin and the cavity was deepened by means of a carbide tipped endcutting bur rotated by a holder held between the fingers and observed through a binocular microscope Both cavities were extended until the pulp was visible through a thin layer of dentin The cavities were tested for electrical recording by insertion of fine platinum wire electrodes as described below Subsequent deepening by very small increments was continued until a satisfactory signal to-noise ratio of the recorded impulses was obtained

Recording of potentials from the two cavities in the canine tooth was obtained by placement of agar gel in the base of each cavity which was then covered with a very small drop of Macrodex The electrodes were then introduced into the cavities and held in place by a micromanipulator The potentials obtained were amplified by a Princeton PAR-4 preamplifier and recorded on 1/4 inch magnetic tape for subsequent frequency analysis During the latter procedure it was possible to count only impulses exceeding a minimum amplitude and in this way avoid appreciable change in the number of active units from which potentials were obtained The necessary initial activity required to observe inhibitory effects was obtained by lowering the extracellular calcium concentration by the application of isotonic sodium citrate solution The potential discharge obtained in this way was found *not* to differ significantly from an active spontaneous discharge when such was present Impulse frequency was averaged during each 30 sec period and plotted as impulses per sec

The term sensory unit is used in this study to include transducer sites which are believed to exist in the dentinal receptor like structure with its fine terminal axon (Arwill 1967), and also sites which may exist within the pulp chamber Impulse frequency refers to potentials from either site, although evidence for a distinction between the activity coming from the two will be presented in a subsequent communication (Scott 1971)

The cervical sympathetic trunk in the neck region was separated from the vagus The trunk was cut and the distal stump placed in contact with silver electrodes connected to a Grass Model S4 Stimulator The nerve was covered with Plastibase (Squibb) insulating gel to prevent drying Square stimulating pulses were used having a duration of 1—2 msec and intensity 6—10 V, frequency was varied between 1 and 10/sec Each period of stimulation lasted 5—10 min

Radioactive tracers, I^{125} and I^{131} were obtained as carrier free sterile isotonic solutions of iodide containing sodium thiosulfate with phosphate buffer pH 7—8 (AB Atomenergi Nyköping Sweden) A volume of 0 1—0 2 microliters solution of I^{12} (40—80 μCi/μl) was placed in the cavity over the pulp horn followed by placement of the recording electrodes and covering the tooth with a small piece of thin plastic film For comparative studies of neighboring tissues a second depot of tracer I^{131} (2—3 μl, 20—40 μCi/μl) was injected in the submucosa close to the apical portion of the tooth The entire preparation was then covered with a further wrap of thin plastic film to reduce moisture loss Final inspection was made to insure that the thermode was satisfactorily controlling the temperature of the tooth which was monitored by the thermocouple

The tracer depots were monitored externally by a scintillation detector connected to a pre amplifier and two single channel pulse height analysers each fed into two scalers (Philips) The experiments were started 7—15 min after the placement of tracer in the dentinal cavity Radioactivity was counted for one min periods The disappearance rate from the dentin was usually monoexponential for 1 hr Three or four subsequent tracer placements could be made in the same cavity Before the experiment the physical background was determined and when double tracers were used, the pulse rates in both recording channels were determined separately for each tracer A part of the gross pulse rate recorded in the I^{131} channel was counted in the I^{125} channel and appropriate corrections were made in the records After correction for total final background the disappearance rate (k value) was calculated for each tracer as follows

$$k = (\log C_1 - \log C_2) / 0\,4343 \; (t_2 - t_1) \quad \text{(Kety 1949)}$$

C_1 and C_2 are the recorded net pulse rates per min of the depot at the time t_1 and t_2 the time interval $t_2 - t_1$ is expressed in min and calculations were made for each 1 min interval The k values thus obtained represent the running average for each interval For further technical details see Bolme and Edwall (1970)

Results

Effect of sympathetic vasoconstrictor nerve stimulation on disappearance rate and impulse frequency

The effects of stimulation of the sympathetic nerve on the disappearance rate of I^{125} and the impulse frequency for three different frequencies of stimulation are shown in Fig 2 Prior to stimulation both k value and impulse frequency had been observed to remain constant over more than ten min The first min of stim with 3/sec Fig 2 A produced a small decrease in k-value which then fell

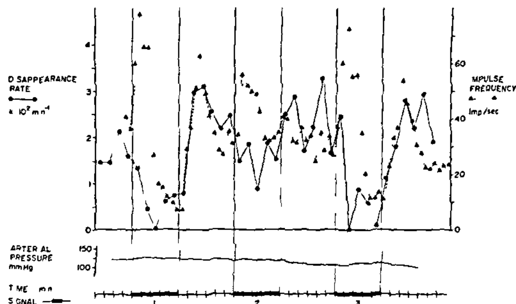


Fig 2 Influence of sympathetic nerve stimulation on disappearance rate and sensory unit impulse frequency Cat young adult 2 4 kg Pentobarbital
1 Stimulation with 10 V 1 msec 3 imp/sec
2 Stimulation with 10 V 1 msec 1 5 imp/sec
3 Stimulation with 10 V 1 msec 6 imp/sec
Each k value represents the running average for a 1 min interval and is plotted in the middle of the interval Impulse frequency is plotted in an analogous way for 30 sec intervals

zero by the third min of stimulation Recovery to about 1/3 the initial value was seen in the fourth and fifth min with a rapid rise to values above control during the first two min of recovery

Changes in impulse frequency during this same period of stimulation started with a rapid rise to double the initial value during the first min This was followed by an equally rapid fall in the second and third min to a value 1/3 the initial frequency and a further slower decrease in the last two min to reach a minimum value about 1/5 of the initial frequency The recovery of frequency following stimulation was as rapid as that of the k value and paralleled it during the first two min of recovery to reach a maximum more than 50 % above the initial value The additional four min of recovery saw both impulse frequency and k value return to their initial values

In contrast to these marked effects stimulation with 1 5/sec (Fig 2 2) showed a decrease in k value during the period of stimulation barely exceeding the random variation There was however an initial increase in impulse frequency of more than 50 % during the first min of stimulation but this had returned to pre stimulation values by the third min and remained at this level for the remainder of the period of stimulation. No meaningful changes in either frequency or k value were seen following stimulation During the third period of stimulation a frequency of

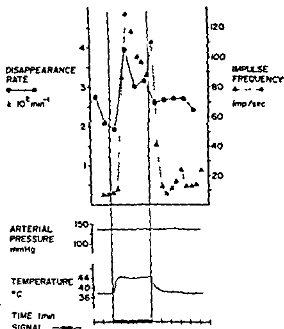


Fig 3 Influence of heat on disappearance rate and impulse frequency Temperature recorded from contact surface between enamel and thermode Cat, mature, 3 2 kg Pentobarbital

6/sec was used and the resultant changes in both impulse frequency and k-value showed a marked similarity to the pattern observed when stimulation at 3/sec was applied

During the three periods of stimulation a slight reduction in femoral blood pressure was observed as shown in Fig 2 Tooth temperature remained constant although this record has not been included The results shown in Fig 2 represent the typical changes observed in 15 experiments, when the response to sympathetic stimulation was a complete or near complete fall of k-value to zero

The effect of warming on disappearance rate and impulse frequency

Reproducable periods of warming were instituted by a rapid increase in the temperature of the thermode to produce a thermal pulse of 6—8° C lasting 4 to 5 min The thermocouple in the tooth indicated a rise time of 1/2 min

In Fig 3 the initial k value of 2×10^{-2}/min was close to that in the previous procedure and in fact was typical of most initial conditions in these experiments Increase of tooth temperature by 6 C the first half min resulted in a rapid rise of k value to almost double its initial value by the end of the first min following which a gradual decline was seen for the rest of the warming period, with little further change after the end of warming

On the other hand the frequency of recorded potentials increased promptly dramatically during the

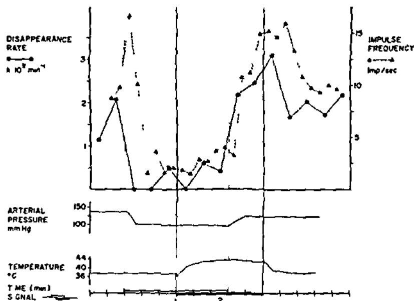


Fig 4 Interaction of warming and sympathetic stimulation on disappearance rate and sensory unit impulse frequency Cat young adult 3 2 kg Pentobarbital
1 Stimulation with 6 V 1 msec 6 imp/sec
2 Heat

which was 15 times the initial frequency Some accomodation of this high frequency was seen during warming but a sharp drop occurred on return to normal temperature when a mean frequency about twice the initial control was reached after 3 min No recognisable change in arterial blood pressure was recorded during or after the period of thermal stimulation Such rapid increases in impulse frequency with temperature have been known from previous studies (Scott 1966) but is was not known to what extent this was the property of the sensory unit and to what extent this was an indication of the effect of vascular reactions The following combined procedure evaluates these alternatives

Interaction of warming and sympathetic stimulation on disappearance rate and impulse frequency

Since sympathetic stimulation results in a decrease in k value and a biphasic response in impulse frequency while on the other hand an increase of tooth temperature evokes an increase in both parameters the experimental interaction of these two procedures should provide information as to the relative effectiveness of each and their mutual interaction In this procedure (Fig 4) sympathetic stimulation was first applied and continued for 6 min at 6/sec Three min after the start of this stimulation, the temperature of the thermode was increased by 6° C and this was continued for 5 min Thus the tooth was subjected first to 3 min of sympathetic

stimulation alone, then to 3 min of both stimulation and warming and finally to 2 min of warming alone

During the initial 3 min period the k value fell to zero in the first min and did not significantly change during the remainder of this period At the same time the impulse frequency underwent a rapid increase to double its initial value in the first half min followed by an equally abrupt fall to near zero value and remained there till the end of this period These changes followed an identical pattern of those seen with 3/sec and 6/sec stimulation in Fig 2 1 and 2 3

The subsequent 3 min period of combined stimulation showed a slow rise in k value During the same period the impulse frequency also showed a slow rise which does not resemble either the further depression seen with continued sympathetic stimulation (Fig 2 1) or the explosive rise in frequency observed at the initiation of warmth alone (Fig 3) While only minor differences are seen in the result from sympathetic stimulation alone and the combined procedure the effect of heat alone shows both greater k values and much greater impulse frequencies than were seen during the period of combined procedure

In the final 2 min when warmth stimulation alone was applied both k value and impulse frequency rose rapidly to supernormal values as though released from a restrictive constraint This is reminiscent of the period following sympathetic stimulation alone (Fig 2 1) as well as the change in both parameters when warmth alone was applied (Fig 3) Following the conclusion of warmth application both parameters fell slowly to their initial values in 3 min, the k value falling somewhat more rapidly than the impulse frequency In this experiment there was a fall in arterial pressure during sympathetic stimulation presumably due to concomitant stimulation of vagal fibres However, in other experiments the arterial pressure was constant during stimulation and the same effect was observed This pattern of response represents the typical result obtained in 6 procedures

Effect of reduced temperature on circulation and impulse frequency

Rapid cooling of the tooth was achieved by sudden circulation of cold water from an ice bath through the thermode Tooth temperature showed a fall from 40° C to 22° C during the period of 4 min as shown in Fig 5 This was accompanied by a rapid decrease in k value which approached zero within the first min of cooling and remained low during the period of cooling although some tendency for release in the last min was seen When the temperature of the thermode returned to normal the k value regained its initial value in two min without appreciable overshoot

Impulse frequency rose to 50 % above initial value during the first min of cooling This was followed by a decrease reaching near zero values at the end of the period of cooling Subsequent return to initial frequency was obtained without overshoot in 3—4 min at a rate slower than that for the k value No change in femoral blood pressure was seen

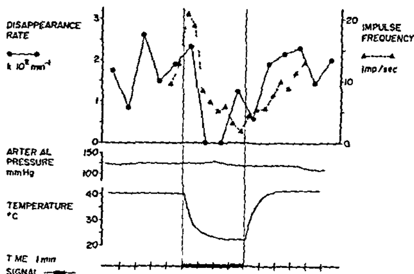


Fig 5 Influence of cold on disappearance rate and impulse frequency Cat young adult 3 2 kg Pentobarbital.

Interaction of cold and sympathetic stimulation

The combined influence of sympathetic stimulation and cooling is shown in Fig 6 Three min after the start of the 6 min period of sympathetic stimulation at 6/sec a 7 min period of cooling was initiated Thus three min of stimulation alone was followed by three min of combined procedure and finally a four min period of cold alone

During the 3 min period of stimulation the change in k value and impulse frequency was similar to that also seen in Fig 2 1 When cold was added to sympathetic stimulation the k value remained depressed although there was a brief increase towards the end of the period Impulse frequency showed tendency towards a small temporary increase at the beginning of cold application but this was followed by a fall to zero where it remained during the last min

The final 4 min period of cold alone showed a marked increase in the k value after the removal of sympathetic stimulation at the beginning of the period so that 3 min later it exceeded the initial value Reduction in this maximum seen in the last min might be influenced by oscillation of the calculated k values The prolonged extreme depression of impulse frequency during cold was only slowly reversed in the last two min

Removal of cold was associated with k values near those found initially although oscillation of the data distorted the record Impulse frequency recovered to near initial values in one min and remained steady thereafter The slow changes of frequency seen during cold resemble those seen with cold alone in Fig 5 Arterial pressure showed a slight reduction during the period of sympathetic stimulation This pattern of responses was consistently obtained in 6 procedures

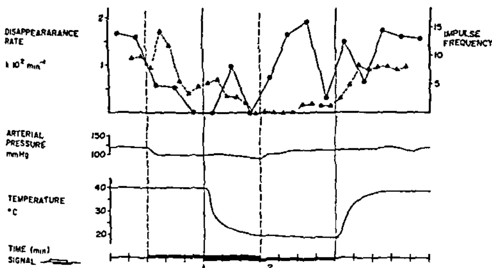


Fig 6 Interaction of cold and sympathetic stimulation on disappearance rate and sensory unit impulse frequency Cat, young adult, 3 2 kg Pentobarbital Disappearance rate (●——●) Impulse frequency (▲- - -▲), imp/sec

1 Stimulation with 6 V, 1 msec, 6 imp/sec
2 Cold

Discussion

In the present study we have investigated the interaction between changes in pulpal microcirculation and the excitability of sensory units in the tooth The determination of the disappearance rate of a small, water-soluble ion from a depot placed in the same cavity as the recording electrode (Mayer 1966, Edwall and Kindlowa 1971) proved to be the most dependable method for providing information of circulatory change in the immediate vicinity of the sensory units The method has the further advantage that it does not interfere with the simultaneous electrical recording of impulse activity in the sensory unit

Decreased pulpal blood flow has previously been observed microscopically during sympathetic nerve stimulation (Taylor 1950 Ogilvie 1967) which has also been found to decrease pulp pressure (Weatherred, Kroeger and Smith 1963) In agreement with these previous observations sympathetic nerve stimulation induced a reduction in k-value to near zero level in the present study as was also reported by Edwall and Kindlova (1971)

In a previous study on tracer disapperance from isolated skeletal muscle it was shown that stimulation frequencies of 2 5 to 10 imp/sec induced a greater reduction in k value than in total blood flow (Bolme and Edwall 1971) Although data from skeletal muscle are not directly comparable with the present data on dental pulp they suggest that the iodide k-value from the dentinal depot in the present was not only dependent on pulpal blood flow but was also influenced by

justments in the capillary section affecting the exchange function Furthermore, the rate of transport (by convection and/or by diffusion) through the dentinal tubules would also limit the disapperance rate to some degree Hindrance by this factor, however, seems to be of little importance since double tracer experiments performed on dog dental pulp showed but small difference in k value between an iodide depot directly injected into the pulp and a similar depot located in a deep dentinal cavity (Edwall, unpublished results) The tracer depot in the present studies was located in the same cavity from which the sensory unit impulses were recorded Therefore, the k-values were primarily influenced by vascular events occuring in close proximity to the neural elements from which the impulses were recorded

The pattern of change in k-value and impulse frequency in response to sympathetic nerve stimulation alone, seen in Fig 2, 4 1, and 6 1, shows the good reproduceability of this effect These examples show a biphasic response in impulse frequency, an initial transient rise and following maximal reduction of k-value, a rapid depression of impulse frequency

Considering first the initial transient rise of impulse frequency, the onset of vasoconstriction would be expected to reduce the availability of metabolic substrates which reach the receptors by diffusion from the capillaries transient increased excitability should result from the restriction of such substrates (Lehmann 1937, Speckmann 1970)

The initial rise in impulse frequency induced by sympathetic nerve stimulation may also be due to a direct effect of released noradrenaline on the sensory units (*cf* Paintal 1964) Furthermore, modifications in pulp pressure may b[illegible]luced by the vascular response to sympathetic stimulation (Weatherred, [illegible]nd [illegible]ith 1963) Such changes may cause a movement of fluid in the [illegible]al [illegible]ibules which would excite sensory units (Brannstrom 1961)

The biphasic change in frequency during sympathetic ner[illegible] *similar to the change seen at the onset of cooling alone* (F[illegible] lowered temperature should act directly to reduce the [illegible] and may thus cause a transient rise in excitability H[illegible] that cooling may produce modifications in pulp pressu[illegible] 1969), which may cause excitation of sensory units ([illegible] known that cooling will excite isolated axons (Shea, [illegible] this effect would be independent of the activity of th[illegible] several possible explanations of the initial rise in ex[illegible] nerve stimulation and cooling

Considering the subsequent period of depression [illegible] sympathetic nerve stimulation this was always acc[illegible] of k-value, indicating a reduction in local blood flow [illegible] hence, a reduced availability of metabolic substrate [illegible] decreased excitability of the sensory units in acc[illegible] on isolated axons (Lehman 1937) and [illegible] asphyxia

In agreement with these considerations, we found that when sympathetic nerve stimulation preceded cooling the application of cold evoked miniature counterparts of the frequency response produced by cold alone (Fig 6)

This was also apparent from the combined experiment in which warming was applied during vasoconstrictive stimulation (Fig 4), the increase of impulse frequency with rise in temperature was influenced by vascular changes as indicated by the k value In this case, a small rise of k value occurred during the combined application and a similar small increase in impulse frequency accompanied it As soon as the vasoconstrictive stimulation was removed a rapid rise of k value and impulse frequency was seen Thus temperature rise during a depressed and essentially constant k value is inadequate to evoke an appreciable increase in impulse frequency This would suggest that excitability of the sensory unit depends on the integrity of its local microcirculation and metabolic activity

In the combined experiment on interaction of cold and sympathetic stimulation (Fig 6), during the period of cold alone, the k value returned to near initial values while the impulse frequency did not show a comparable change This illustrates that the depression of impulse frequency by cold is not merely an expression of the influence of the circulation but may also depend on some other more basic property

However the decreased impulse frequency evoked by either cold or sympathetic nerve stimulation may be directly or indirectly dependent on the same causal factor It can be assumed that the ultimate effect of both cold and reduced blood circulation is a decrease of metabolic activity which may decrease receptor excitability by modification of the electrolyte concentration gradient across the excitable membrane (Lehmann 1937)

Quantitative differences have been found in the changes of both k value and impulse frequency evoked in individual animals and are most probably explained by variations in pulp size dependent on age Examination of pulp diameter by X ray films has shown that in young adult cats the pulp diameter may exceed 60 % of the outside diameter of the tooth whereas in mature cats with well rounded tips of the canine teeth, the pulp diameter was only about 25 % of the outside diameter In the latter type of teeth it was found that vasoconstrictor nerve stimulation (3—10 imp/sec) produced a marked increase in nerve impulse frequency without a subsequent decrease This appears to represent a prolongation of the initial increase in impulse frequency which was seen at the start of such stimulation in young adult teeth and implies a submaximal response to sympathetic stimulation Examination of changes in k value during stimulation of these teeth shows only a partial decrease from resting value and is in agreement with the above hypothesis

Afferent neurons from tooth pulp have been reported only to give rise to the sensation of pain in human subjects (Sicher 1966) The results of the present study indicate that changes in pulp microcirculation which may occur simultaneously with some other form of excitation may serve to modulate the resulting sensory experience

This investigation was supported by grants from the Swedish Medical Research Council (B 70 24X 816 B 71 24X 817 06 B 71 24 P 2625 03), Karolinska Institutet and National Inst of Health Bethesda Md USA (Grant DE 02062)

We express our appreciat on for the close cooperation and advice from Docent Aage Moller

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Acta physiol. scand 1971. 82. 567—570
From the Department of Physiology I, Karolinska Institutet, Stockholm, Sweden

Lipids of the Sympathetic Nerve Trunk Vesicles. Comparison with Adrenomedullary Vesicles

By

HUGO LAGERCRANTZ

Received 18 February 1971

Abstract

LAGERCRANTZ, H *Lipids of the sympathetic nerve trunk vesicles Comparison with adrenomedullary vesicles* Acta physiol scand 1971 *82* 567—570

The phospholipid and cholesterol contents of isolated nerve trunk vesicles were analyzed in a preparation obtained [illegible] storage particles Th[illegible] of this preparation [illegible] ever, lysolecithin, w[illegible] found only in trace amounts in the nerve vesicle preparation The results support earlier findings of biochemical similarities between the water insoluble phase of the two types of amine storage vesicles

Biochemical similarities between the water insoluble matrix of sympathetic nerve trunk vesicles (N vesicles) and that of adrenomedullary vesicles (A vesicles) *e g* the dopamine β hydroxylase/protein and cholesterol/phospholipid ratios, have previously been reported (Helle Lagercrantz and Stjarne 1971) In the present study the lipids of the two types of vesicles are further compared

The subject is of particular interest in view of the possible role of the lipids for catecholamine (CA) storage and release as suggested by Euler *(1916) There* are several reports on the lipids of the A vesicles (Blaschko *et al* 1967, see Smith 1968) However the lipid composition of the N vesicles has not been studied since preparations of necessary purity have not been available In this laboratory a method has been worked out to prepare a highly purified fraction of N vesicles (Lagercrantz Klein and Stjarne 1970) Thus it has become possible to compare the lipid composition of A and N vesicles However interpretation of the results is complicated by the fact that the N vesicle preparation is obtained from nerve trunks and may differ in biochemical composition from the N vesicles in the terminal varicosities

Methods

Isolation of subcellular fractions The present preparative method has earlier been by Lagercrantz Klein and Stjarne (1970) and is slightly modified according to Lagercrantz (1971) Bovine splenic nerves were homogenized by the Ultra T

and the homogenate was centrifuged at $600 \times g$ for 10 min and then at $10,500 \times g$ for 10 min The supernatant (SN) was layered on continuous sucrose D_2O gradients (0 25 M—0 6 M) with a step in the bottom of the tubes (1 3 M) Three visible bands were formed after ultra centrifugation which were [illegible]emoved by a pipette diluted to isotonicity and named F I, F II and F III respectively In a few experiments F III was further purified by centrifugation at $15,000 \times g$ for 10 min after dilution to isotonicity All fractions were spun down in the ultra centrifuge before lipid extraction

Lipid extraction Lipids were extracted essentially as described by Folch *et al* 1951 The pellets (F, I F II, F III) and the SN were resuspended in 3 ml of distilled water 2 1/2 ml was mixed with about 25 ml chloroform methanol (2 1) in separatory funnels 5 ml 0 9 % NaCl was added After storage in the refrigerator for at least 12 hrs the bottom phase was removed and evaporated under nitrogen

Separation and analysis of the phospholipids [illegible] ed and the lipids separated by thin layer chromatography [illegible] methanol formic acid (2 1) and activated [illegible] with chloroform methanol acetic acid wat[illegible] which were detected with iodine were removed and eluated as described by Skipski (1964) The phosphorus was determined according to Bartlett (1959) The recovery of phosphorus applied on the plates was 94 7 ± 4 1 % (SEM)

The phospholipids were identified by comparison with standard preparations Phosphatidyl ethanolamine and phosphatidylserine could also be detected after spraying the plates with nin [illegible] Phosphatidylethanolamine, lecithin [illegible] nd in the [illegible]

Protein was determined with the Folin phenol reagent according to Lowry *et al* (1951)

Materials Kiselgel H was obtained from Merck (Darmstadt, West Germany) The phospholipids standards were kindly supplied by Dr S Hammarstrom, Dept of Medical Chemistry Royal Veterinary School Stockholm

Results

Subcellular fractions Three subcellular fractions were obtained as described under [M]ethods F I was the microsomal band with the highest activity of glucose 6-phosphatase activity F III consisted of about 40 % of NA noradrenaline storage particles No glucose-6-phosphatase activity was found The main contaminants seemed to be microtubular and mitochondrial fragments By further purification of F III most of the cytochrome oxidase activity was removed (see Klein and Lagercrantz 1971) F II also had a high content of N vesicles but it was somewhat contaminated with microsomes The fractions have earlier been biochemically and morphologically characterized (Lagercrantz Klein and Stjarne 1970 and Klein and Thureson-Klein 1971)

Subcellular distribution of lipid phosphorus and cholesterol Table I shows the content of lipid phosphorus and cholesterol per mg protein and the percentage distribution between the 3 fractions The microsomal fraction (F I) had the highest content of both cholesterol and phospholipids

Phospholipids The phospholipids were further analyzed by thin layer chromatography The results are shown in Table II Phosphatidylethanolamine and lecithin were found to be the main phospholipids in all fractions Only minor differences could be seen between the microsomal and the N vesicle fractions F I contained relatively more sphingomyelin Only small amounts of lysolecithin could be detected in the N vesicle fraction (F III) but more than in the other fractions As

TABLE I Subcellular distribution of lipid phosphorus and of cholesterol between the density gradient fractions Means of 5 expts ± SEM

Fraction	Lipid P % of total	μmoles/mg protein	Cholesterol % of total	μmoles/mg protein
F I	31 7	1 09 ± 0 08	29 2	0 75 ± 0 04
F II	16 8	0 74 ± 0 08	15 3	0 39 ± 0 14
F III	13 4	0 78 ± 0 05	11 1	0 35 ± 0 05
% recovery	61 9		55 7	

described earlier, F III was further purified in some experiments but the lipid analyses did not show any significant differences from those of the whole F III except that less cardiolipin was found

The analytical methods used in the present study were essentially the same as those of Blaschko *et al* (1967) in their analysis of the lipids of A vesicle and the present values for lipids of A vesicles were in agreement with their results

Discussion

Some biochemical similarities between the water insoluble phase of the N and that of the A vesicles have previously been reported (Helle, Lagercrantz and Stjärne 1971) Considering that the N vesicle preparation was only half as pure as the A vesicle preparation the dopamine β hydroxylase activity per mg protein was found to be about the same in the two types of vesicles, while only about a third of the chromogranin amount/mg protein in the A vesicles was found in the N vesicles The phospholipid/protein ratio was found to be 0 78 μmoles/mg in the N vesicle preparation while the corresponding value for the A vesicles was found to be 0 45 (originally 2 83 μmoles/mg N according to Blaschko *et al* 1967) This difference corresponds to the higher percentage of water insoluble protein in the N vesicles (80 %) than in the A vesicles (20 %) (Helle Lagercrantz and Stjarne 1971) It also corresponds to the relative larger volume of membrane in the N vesicles assuming that the membrane has about the same thickness in both vesicles and that the N vesicles are considerable smaller (*cf* Klein and Thuresson Klein 1971)

TABLE II Phospholipids separated by thin layer chromatography Figures represent the percentage of the total lipid phosphorus recovered from the plates (means ± SEM of 6 expts)

	F I	F II	F III
Phosphatidic acid and cardiolipin	2 6 ± 0 9	1 2 ± 0 2	4 9 ± 0 4
Phosphatidylethanolamine	28 4 ± 2 3	31 8 ± 1 3	30 8 ± 2 7
Phosphatidylserine and phosphatidylinositol	11 8 ± 1 0	6 8 ± 2 6	7 1 ± 1 4
Lecithin	31 6 ± 2 2	38 4 ± 1 7	36 6 ± 2 2
Sphingomyelin	26 3 ± 0 3	23 1 ± 1 1	17 6 ± 2 8
Lysolecithin	1 1 ± 0 6	0 7 ± 0 4	3 1 ± 0 8

The further analysis of the phospholipids of the N vesicles preparation by thin layer chromatography showed certain similarities between the two types of vesicles (*cf* Blaschko *et al* 1967), with phosphatidylethanolamine and lecithin as the major constituentes However, only small amounts of lysolechithin was found in the N vesicle preparation On the other hand it contained a correspondingly higher amount of lecithin

The occurrence of lysolecithin in A vesicles seems to be well established (see Smith 1968) This study has shown that the N vesicles probably lack significant amounts of lysolecithin Our finding is probably not due to failure to extract lysolecithin from the original fractions since about 80 % of all the phosphorus was extracted and since about 15 % lysolecithin was found in A vesicles with the present method

Lysolecithin has been ascribed an important role for the release of CA from adrenal medulla (see Smith 1968) On the basis of the present results it is not possible to exclude a similar function for lysolecithin in sympathetic nerves The possibility remains that lysolecithin might be formed in the vesicles of the nerve terminals as a result of *e g* lysosomal phospholipase acting on the vesicle membranes as suggested by Winkler and Smith (1968)

This study was supported by the Swedish Medical Research Council project no K70 14X 2479 03A and by Stiftelsen Lars Johan Hierta I wish to thank Drs L Nystrom and B Almé Dept of Medical Chemistry for valuable discussions and Mrs I Dahlin for her highly competent technical assistance

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From the Department of Pharmacology University of Goteborg Sweden

Formation of ¹⁴C-Octopamine from ¹⁴C-Tyramine in the Mouse: A Dose Dependent Relationship

By

Bertil Waldeck

The sympathomimetic amine, tyramine (TA), has been shown to be taken up into the adrenergic nerves, where it is rapidly converted to octopamine (OA) by the enzyme dopamine β hydroxylase (Carlsson and Waldeck 1963) This OA can be released on stimulation of the adrenergic nerve (Fischer *et al* 1965) Recently OA has been shown to be a normal constituent of the sympathetic nerves of the rat (Molinoff and Axelrod 1969) From this point of view the study of the metabolism of TA appears to be the more important

During the last few years ^{14}C labelled TA with an increased specific activity has become available thus making it possible to study the metabolism of TA at lower concentrations We soon found that the relative yield of ^{14}C-OA from ^{14}C TA increased markedly when the dose decreased The present investigation was undertaken in order to study this dose dependance in more detail

Female mice weighing about 20 g were randomly divided into groups of six All experiments were performed at an ambient temperature of about 25° C The tissues to be analysed were extracted in perchloric acid after which ^{14}C OA and ^{14}C-TA were isolated by ion exchange chromatography This analytical procedure has been described in detail elsewhere (Almgren *et al* 1965 The drugs p-hydroxyphenyl ethylamine 2 ^{14}C HCl (50 mc/mmole) and tyramine HCl were used All data are given as hydrochlorides

Mice were given ^{14}C TA 10 μg/kg i v alone or with cold TA added to give a total dose of 50 100 and 200 μg/kg respectively The animals were killed 30 min after the injections The contents of ^{14}C-OA and ^{14}C TA in the heart and femoral muscle were determined and the total amount of amines (labelled + unlabelled) was calculated

In the heart the relative yield of ^{14}C OA decreased as the dose of ^{14}C TA was increased (Fig 1) Thus when the dose of ^{14}C TA was increased from 10 to 200 μg/kg *i e* 20 times ^{14}C OA increased only 3 times In the femoral muscle the relative yield of ^{14}C OA was less than in the heart but appeared to increase more linearly at least in the dose range below 100 μg/kg of ^{14}C TA In fact from 0—64 μg/kg

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From the Depar nent of Physiology, University of Goteborg, Sweden

Studies on Cardiac Ventricular Receptors

A preliminary report

By

Bengt Öberg and Peter Thorén

Recent studies have shown that a sudden reflex slowing of the heart sometimes appears during a rapid blood withdrawal in cats (Öberg and White 1970) This reflex bradycardia was triggered from receptors with their afferent connections joining the vagus in the cardiac nerves and located in the heart ventricles (Öberg and Thoren 1970) In the present study these receptors were studied in more detail with respect to their discharge characteristics

Cats anesthetized with chloralose were thoracotomized, the pericardium was opened and snares placed around the aorta and the right coronary artery The right cardiac nerve was freed to be accessible for electrical stimulation but elsewise left intact Thin filaments from the right cervical vagus were placed on an electrode for recordings of impulse activity both on an oscilloscope and by a spike counter device on a Grass Polygraph recorder The right vagus was cut below the entrance of the cardiac nerve This eliminated almost completely the afferent traffic from pulmonary inflation receptors which greatly facilitated the identification of, and recording from cardiac afferents — Heart rate arterial blood pressure and left ventricular pressure were recorded with conventional techniques on the Grass recorder One femoral artery was cannulated for bleeding purposes In some experiments the heart ventricles were placed in a cardiograph for measurement of ventricular volume changes

Cardiac receptor afferents were first identified by the increased impulse activity following a shortlasting partial occlusion of the aorta Fig 1 shows recordings of left ventricular pre sure arterial blood pressure spike frequency in two cardiac afferents and neurogram recorded at time A B and C respectively before and during a progressive aortic occlusion The two afferents have almost identical spike heights Both receptors could be excited al o by mechanical stimulation of a localized apical area of the left ventricle From recording of the potentials evoked by electrical stimulation of the right cardiac nerve the conduction velocities were calculated to be 0 8 and 0 9 m/sec respectively indicating non medullated C-fibres They display a very low resting spontaneous activity with no clearcut cardiac rhythm (neurogram A) With aortic occlusion their activity increases though first when the obstruction is severe enough to cause a substantial rise of ventricular diastolic pressure, and they now display cardiac rhythm neurogram B When the aorta is totally occluded, their discharge becomes continuous (neurogram C)

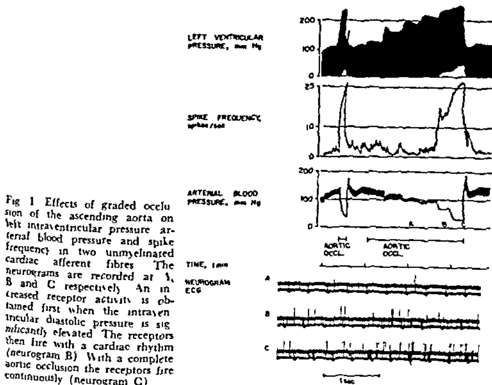


Fig 1 Effects of graded occlusion of the ascending aorta on left intraventricular pressure arterial blood pressure and spike frequency in two unmyelinated cardiac afferent fibres The neurograms are recorded at A, B and C respectively An increased receptor activity is obtained first when the intraventricular diastolic pressure is significantly elevated The receptors then fire with a cardiac rhythm (neurogram B) With a complete aortic occlusion the receptors fire continuously (neurogram C)

Measurements of changes in ventricular volume and in heart rate during aortic occlusion revealed that the increased ventricular diastolic pressure, as expected, was correlated to a clear increase of ventricular end diastolic volume and that this ventricular distension was regularly accompanied by a reflex bradycardia The studied receptors therefore seem to be activated by such distension and then initiate a reflex slowing of the heart They were accordingly excited also by other procedures causing increased ventricular end diastolic volume such as saline infusion or elevations of arterial pressure by i v adrenaline or carotid occlusion

An increased receptor activity was also seen when the nutritional supply to the heart was compromised *e g* by asphyxiation of the animal or by occlusion of one coronary artery This increased receptor activity displayed at least initially a cardiac rhythm and appeared first when volume measurements revealed a marked ventricular dilatation These findings are compatible with the classification of the receptors as mechanoreceptors rather than chemoreceptors

As earlier described (Öberg and Thorén 1970) the receptors could often be activated by rapid bleeding probably due to receptor deformation when the ventricles were contracting vigorously around an almost depleted chamber They were also activated by various drugs, local nicotine application in the pericardial cavity thus stimulated about 30 % of the studied receptors The same receptors were activated also by stroking the ventricular epicardial surface Other ventr-

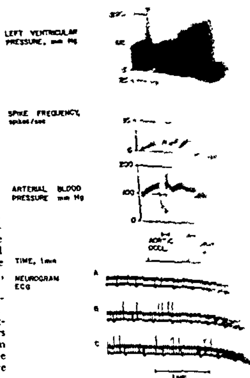


Fig 1 Effects of graded occlusion of the ascending aorta on left intraventricular pressure, arterial blood pressure and spike frequency in two unmyelinated cardiac afferent fibres The neurograms are recorded at A, B and C respectively An increased receptor activity is obtained first when the intraventricular diastolic pressure is significantly elevated The receptors then fire with a cardiac rhythm (neurogram B) With a complete aortic occlusion the receptors fire continuously (neurogram C)

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receptors responded to i.v. injected nicotine and required a more forceful mechanical squeezing of the ventricles to become activated. This discrepancy with regard to receptor response to nicotine administered by various routes is therefore probably explained by their localization in superficial and deep layers of the ventricular myocardium rather than being a manifestation of two functionally different receptor groups. The receptors also responded to intravenous injections of veratrum alkaloids and are therefore responsible, at least partly, for the Bezold—Jarisch reflex.

The adequate stimulus for the described receptors thus seems to be a distorsion of the ventricular wall, whether caused by distension or squeezing of the myocardium. The receptors then discharge with a cardiac rhythm or, if the ventricular deformation is severe, continuously. The reflex circulatory effects then produced are, besides the bradycardia, not quite clear. Since, however, electrical stimulation of unmyelinated afferents in the cardiac nerves produces bradycardia, hypotension and vasodilatation (Öberg and Thorén, to be published), it is likely that the present receptors when activated give rise to similar circulatory effects. They might therefore be ascribed a *protective function aiming at reducing the work of an overloaded heart* by inducing reflex bradycardia and hypotension, a type of reflex mechanism suggested already by Daly and Verney (1927). They also seem to be responsible for inducing a sudden "vaso-vagal syncope"-like reaction with a reflex bradycardia if the ventricles are "squeezed empty" as a result of poor diastolic filling (*cf.* Henry 1955).

Ventricular receptors, signalling in thin vagal afferents and in many respects resembling those presently described, have been reported in cats (Jarisch and Zotterman 1948) and in dogs (Sleight and Widdicombe 1965). A different type of ventricular endings with non-medullated afferents, extremely sensitive to mechanical stimulation and therefore probably very superficially located, possibly in the epicardium, have also been described by Coleridge, Coleridge and Kidd (1964) and Sleight and Widdicombe (1965).

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